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OLIGODENDROCYTE APOPTOSIS IN VITRO: RELATIONSHIP TO INSULIN AND INSULIN-LIKE GROWTH FACTOR-I WITHDRAWAL, NEW PROTEIN SYNTHESIS AND MITOCHONDRIAL MEMBRANE POTENTIAL

by

SAI LATHA SHANKAR

Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia

November 1998

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in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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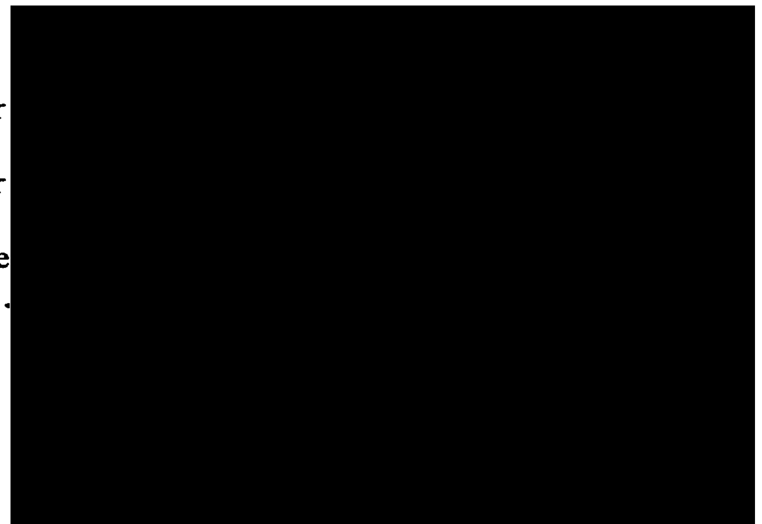

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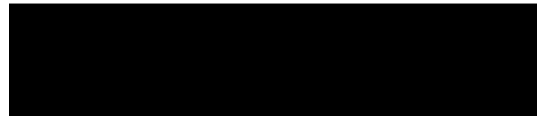

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Dedication

This thesis is dedicated to

Bhagawan Sri Sathya Sai Baba, my parents Ramaa and Sankara Loganathan and sister Vishali.

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Abstract

This thesis describes an *in vitro* model established to study whether withdrawal of insulin, insulin-like growth factor - I (IGF-I) and serum causes apoptosis in oligodendroglial cells. *In vitro*, oligodendrocyte-type 2 astrocyte progenitor cells (O-2A) can develop into either an oligodendrocyte or a type-2 astrocyte. In the present study, O-2A progenitor cells derived from the rat cerebral cortex were differentiated into oligodendrocytes in the presence of insulin and IGF-I over 16 days. Antibodies to O-2A lineage specific developmental markers were used to characterize the cells.

Placement of the cells into insulin, IGF-I and serum withdrawn media for 24h led to decreased numbers of intact nuclei and cells. The insulin and IGF-I deprived cells showed extensive process fragmentation but displayed intact cell bodies. Chromatin fluorescence of trophically deprived cells revealed nuclear stigmata typical of apoptosis. *In situ* nicked end labeling and gel electrophoresis revealed DNA fragmentation beginning at 6 h after insulin, IGF-I and serum withdrawal and peaking between 18 to 24 h.

(-) -Deprenyl, a monoamine oxidase- B inhibitor markedly increased the survival of insulin, IGF-I and serum withdrawn oligodendrocytes. Experiments with general inhibitors of cytochrome P450 enzymes revealed (-) -desmethyldeprenyl as the active metabolite that mediated the anti apoptotic effects of (-) -deprenyl. The anti-apoptotic effects of (-)-desmethyldeprenyl was blocked by actinomycin and cycloheximide showing that (-)-desmethyldeprenyl required new protein synthesis for its anti-apoptotic action. (-) -Deprenyl and (-) -desmethyldeprenyl significantly increased the levels of myelin basic protein and proteolipid protein relative to that of insulin, IGF-I and serum withdrawn cells. (-) -Desmethyldeprenyl also prevented the decrease in mitochondrial membrane potential observed by insulin, IGF-I and serum withdrawal. These findings demonstrate for the first time that compounds like (-) -desmethyldeprenyl can maintain the survival of oligodendrocytes and their precursors in conditions of trophic insufficiency.

List of Abbreviations and Symbols

$\Delta\psi_M$	Mitochondrial membrane potential
AIF	Apoptosis initiating factor
AM	Amphetamine
AST	Astrocyte
ATP	Adenosine triphosphate
BM	Base Media
BM-D9	Base media with 10^{-9} M (-) -deprenyl
BM-Ds9	Base media with 10^{-9} M (-) -desmethyldeprenyl
CMTMR	Chloromethyl tetramethylrhodamine
CNPase	Cyclic nucleotide phosphorylase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Cyt. C	Cytochrome C
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminiscence
EDTA	Ethylene diamine tetracetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Galactocerebroside

IGF-I	Insulin-like growth factor-I
IGFR-I	Insulin-like growth factor-I receptor
IM	Insulin+IGF-I Media
IM-D9	Insulin+IGF-I media with 10^{-9} M (-) -deprenyl
IR	Insulin receptor
ISEL	In situ end labeling
MA	Methamphetamine
MAO-B	Monoamine oxidase-B
MBP	Myelin basic protein
Meta.	Metapyrone
MS	Multiple sclerosis
NGF	Nerve growth factor
O-2A	Oligodendrocyte-type2 astrocyte
ODM	Oligodendrocyte differentiating medium
OL	Oligodendrocyte
P. But.	Piperonyl butoxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PLP	Proteolipid protein
Proad.	Proadifen
PROL	Proligodendrocyte

PTP	Permeability transition pore
SDS	Sodium dodecyl sulfate
TdT	Terminal deoxynucleotidyl transferase

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1.0 Introduction

1.1 Overview

Glial and neuronal numbers in the mammalian central nervous system (CNS) are determined as a result of the progression of cells through a series of temporal stages including induction, proliferation, migration, differentiation, degeneration and death. Cell degeneration and death are critical processes, which ultimately serve to counterbalance proliferation and to decide the total numbers of cells in any specific region of the CNS. It is thought that cell degeneration and death mechanisms occur in order to eliminate functionally inappropriate, damaged or abnormal cells that could impair or interfere with the operation of a particular neural structure. For more than forty years, cell death has been recognized to be central to nervous system development (Glucksmann, 1951; Saunders, 1966). Cell degeneration and death has been classified as belonging to three major types, phylogenetic, morphogenetic and developmental. Phylogenetic cell death is the death of cells that were once characteristic of an evolutionary ancestor but became functionally inappropriate over development. Morphogenetic cell death serves as a process that patterns the nervous system. During the development of the CNS, cells are generated in excess and during the so called critical developmental period, functionally inappropriate cells degenerate and are eliminated. This type of cell degeneration and death has been variously termed as developmental, physiological, histogenetic, programmed or naturally occurring cell death (Cowan, 1973; Oppenheim, 1991).

Inadequate survival signals have been suggested to be one of the predominant initiating events for the mechanisms of developmental cell death in the CNS (Raff, 1992).

The relationship between survival signals and developmental cell death in the CNS was first recognized by the pioneering studies of Levi-Montalcini and Hamburger on neuron-target cell interactions and developmental neuronal death. The exchange of trophic signals between cells is now known to be a major factor in the determining which cells survive and which cells degenerate and die during development. An understanding of trophic signaling in nervous system development now appears to be relevant to the pathogenesis of some glial and neuronal degenerative diseases (Tatton *et al.*, 1997).

Cell death is now recognized to proceed by three different processes, apoptosis, necrosis and atrophy (Ellis *et al.*, 1991). The study of Kerr *et al.* (1972) was one of the first to differentiate apoptosis from necrosis on the basis of differences in cytology. Cell swelling and breakdown of the external membranes of the cell were known to be typical features of necrosis. Using electron microscopy, Kerr and his colleagues described nuclear degradation, particularly chromatin condensation, in cells that retained intact cell membranes. Because the cells underwent shrinkage rather than swelling, they termed the process 'shrinkage necrosis'. Shrinkage necrosis was subsequently termed apoptosis. Subsequently, Kerr's colleague, Wyllie and his associates, recognized that endonuclease-mediated nuclear DNA cleavage is a principal identifying feature of nuclear degradation in apoptosis (Arends *et al.*, 1990). The third cell death process, atrophy, involves the gradual shrinkage and progressive dysfunction of cells that has been found to accompany nervous system aging (Finch, 1993). Apoptosis can occur over hours or days while necrosis occurs within minutes to hours of insult presentation. Atrophy on the other hand is protracted and occurs over months or years.

Naturally occurring apoptosis is difficult to study since apoptotic nuclei are short-lived and the cells enter apoptosis in highly desynchronized manner. Only a small proportion of cells are in the apoptotic process at any time point so that biochemical or molecular methods are faced with the difficulty of detecting changes that accrue from only a few apoptotic cells mixed with large numbers of normal cells. Accordingly. models in which cells enter apoptosis in a highly synchronized manner have been extremely valuable in studying the mechanisms underlying the apoptotic process. Models in which trophic support is suddenly withdrawn from cultured cells have proven to be particularly valuable. Furthermore, the study of apoptotic mechanisms has largely been facilitated by the development of light microscopic techniques that detect nuclear DNA cleavage and/or chromatin condensation *in situ* (see Tatton *et al.*, 1997). The *in situ* methods allow the relatively small proportion of apoptotic cells to be directly examined while using their unaffected neighbors as controls (see Wadia *et al.*, 1998).

Most studies of nervous system apoptosis have focused on nerve cells. Relatively little attention has been paid to glial cell apoptosis. Glia, derived from the Greek word for glue, were initially thought of as cells which simply provide a framework to hold neuronal cells together. It is now known that glia subserve a number of roles in nervous system operation and that those roles are considerably more critical than just holding the neurons together (Travis, 1994).

One of those roles involves the facilitation of action potential transmission by the myelination of nerve cell axons. In the mammalian central nervous system, myelination is carried out by oligodendrocytes that synthesize myelin, a lipid rich biological membrane, which forms into multilamellar sheaths. In 1928, Rio Hortega using innovative metal

impregnation techniques based on silver carbonate or silver nitrate first described the presence of oligodendrocytes in the CNS. Astrocytes are involved in a number of functions, most notably as guides for neuronal migration, process outgrowth and the synthesis and provision of a number of neurotrophic factors including insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3). Microglia, the third glial cell type, are bone marrow derived macrophages which function as immunocompetent cells in the CNS. Microglial cells constitutively express receptors for Fc portion of immunoglobulin. Binding of Fc to its receptors which is specific antibody dependent allows the microglial cells to adhere to the target cells and mediate phagocytosis.

1.2 Oligodendrocyte Differentiation

Oligodendrocytes are generated postnatally from undifferentiated pleuripotential cells. Two major alternate cell types are generated from these pleuripotent cells: oligodendrocytes and type-2 astrocytes. Cells expressing the antigenic phenotype of oligodendrocytes are first detected around the time of birth in rats while the first appearance of type-2 astrocytes occurs at 7-10 days after birth (Abney *et al.*, 1981; Miller *et al.*, 1985). In the rat CNS, three main oligodendroglial cell types have been identified. They are the undifferentiated oligodendrocyte - type 2 astrocyte (O-2A) progenitor cells, the partially differentiated proligodendrocytes (PROLs) and the fully differentiated oligodendrocytes (OLs).

Most of the information on oligodendrocyte ontogeny has been obtained from *in vitro* studies using dissociated cultures from neonatal rat or mouse cortex or rat optic

nerve (Gard and Pfeiffer, 1989; Norton and Farooq, 1993). Primary cultures of oligodendroglial cells provide a powerful model system to study the regulation of differentiation, function and death. Cultured oligodendrocytes mimic their *in vivo* counterparts in that they synthesize myelin specific proteins and also produce myelin-like membranes (Knapp *et al.*, 1987; Szuchet *et al.*, 1986). Oligodendrocytes *in vitro* can be maintained in chemically-defined media containing low serum which allows for the study of the actions of specific molecules on the cells.

1.2.1 Oligodendrocyte-type 2 astrocyte (O-2A) progenitors

The characterization of the O-2A lineage cells in the last decade has facilitated an understanding of glial cell development. Several studies have shown that O-2A progenitor cells are bipotential and can undergo differentiation along two lines into either oligodendrocytes or type-2 astrocytes. The O-2A cells display a high degree of developmental plasticity and their differentiation can be manipulated by placement into a defined *in vitro* environment (Saneto and de Vellis, 1985). Extracellular signaling molecules like serum and trophic factors and cell-intrinsic factors regulate the sequential differentiation of the O-2A progenitor cells. Culture of O-2A progenitors in low serum conditions causes them to differentiate into oligodendrocytes. Higher concentrations (10% or more) of fetal calf serum induce their differentiation into a type-2, process bearing astrocytes (Raff *et al.*, 1983; Temple and Raff, 1985).

Developmental studies have shown that O-2A progenitor cells originate in the subventricular zone of the forebrain, the subependymal layers of the 4th ventricle and in the ventral spinal cord (Curtis *et al.*, 1988; LeVine and Goldman, 1988; Pringle and

Richardson, 1993; Reynolds and Wilkin, 1988; Warf *et al.*, 1991). Prior to migrating from those sites, the cells proliferate. In the rat brain, an initial population of O-2A progenitors of about 5×10^5 cells generates about 6×10^7 oligodendrocytes. O-2A progenitor cells are highly motile and migrate from the germinal zones of origin into widespread regions of the developing CNS including the subcortical white matter, cerebellum, spinal cord and the optic nerve (Small *et al.*, 1987; Warf *et al.*, 1991). After migrating to their final destinations, the O-2A cells differentiate into PROLs and OLs capable of initiating myelination.

1.2.2 Maturation of O-2A Progenitors

The bipolar O-2A progenitor cells differentiate into intermediate, multipolar PROLs (Gard and Pfeiffer, 1990). The PROLs further differentiate into complex, multipolar OLs (Gard and Pfeiffer, 1989; Raff, 1989).

Initially, the cells of the O-2A lineage were characterized only on the basis of their morphologies. Later studies showed that different cells in the oligodendrocyte lineage express specific developmental marker proteins (Raff *et al.*, 1979; Reynolds and Wilkin, 1988). The bipolar O-2A progenitor cell can be recognized by antibodies against three surface tetragangliosides, A2B5, LB1 and GD3 (Levi *et al.*, 1987; Raff *et al.*, 1983; Reynolds and Wilkin, 1988). The O-2A progenitor cells do not express the astrocyte specific intermediate filament protein, glial fibrillary acidic protein (GFAP). PROLs begin to express surface galactosphingolipids like galactocerebroside (GC) (Raff *et al.*, 1979) and cyclic nucleotide phosphorylase-I (CNPase-I) but do not express A2B5, LB1 or GD3 (Mirsky *et al.*, 1980). A few *in vitro* studies have reported that the differentiation

into PROLs is also marked by the appearance of cell surface reactivity with a monoclonal antibody O4 prior to the expression of GC and CNPase-I (Schachner *et al.*, 1981; Bansal *et al.*, 1992). OLs are immunopositive for GC and CNPase-I but also express reactivity to myelin basic protein (MBP) and proteolipid protein (PLP) (Dubois Dalcq, 1987; Zeller *et al.*, 1985). As well as MBP and PLP, OLs express a number of other myelin related proteins including myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG) (Dubois Dalcq *et al.*, 1986). MBP forms 30 to 40% of CNS myelin protein while PLP forms 50% of the protein mass of myelin. MBP and PLP are present in the complex processes of mature OLs.

In order to study apoptosis in the oligodendrocytic and type-2 astrocytic lineage, I developed pure cultures of O-2A progenitor cells using a modified differential shake-off procedure (McCarthy, 1980). The O-2A progenitor cells were differentiated in the presence of insulin and IGF-I. Individual cell types in the lineage were identified by combining immunocytochemistry for antibodies against cell specific marker proteins with morphologic identification criteria. The approach allowed comparisons of apoptotic events and the effects of anti-apoptotic agents in the different cell types.

1.2.3 Trophic Factor Regulation of Oligodendrocyte Differentiation

The differentiation of O-2A progenitor cells into OLs is influenced by a number of polypeptide trophic factors including PDGF, basic fibroblast growth factor (bFGF), CNTF, NT-3, insulin and IGF-I (Barres and Raff, 1994). The trophic factors can be synthesized by either neuronal axons or astrocytes or by both. Astrocytes have been shown to synthesize PDGF, IGF-I and NT-3 both *in vitro* and *in vivo*. CNTF has been shown to be synthesized by both neurons and astrocytes (Henderson *et al.*, 1994; Seniuk-Tatton *et al.*, 1995). Barres and Raff (1994) have suggested that the synthesis and release of the protein trophic factors by astrocytes occur as a consequence of stimulation by electrical activity dependent axonal signals.

Trophic factors like CNTF induce the differentiation of O-2A cells into type-2 astrocytes while PDGF induces O-2A cell differentiation into OLs (Lillien and Raff, 1990). In the presence of PDGF, the O-2A progenitor cells generally undergo about 8 divisions before synchronously differentiating into OLs (Noble *et al.*, 1988; Raff *et al.*, 1988; Richardson *et al.*, 1988). Factors like bFGF are mitogenic for the O-2A progenitor cells and keep the O-2A cells in the mitotic cycle thereby preventing their differentiation into OLs. In contrast, insulin and IGF-I have been shown to induce irreversible OL differentiation (McMorris and Dubois Dalcq, 1988; Raff *et al.*, 1983).

1.2.4 IGF-I, Insulin and Oligodendroglial Proliferation and Differentiation

IGF-I and insulin are structurally and functionally related polypeptides that regulate a number of cellular processes like proliferation, differentiation and survival. The CNS was thought to be insulin and IGF-I independent since serum insulin and IGF-I do not cross the blood-brain barrier. However, the findings of the presence of insulin and insulin receptors in the rat brain radically changed the view that the CNS was insulin independent (Baskin *et al.*, 1983; Havrankova *et al.*, 1978; Kappy *et al.*, 1984). It is now known that IGF-I and II, their receptors, and IGF binding proteins (IGFBPs; secreted proteins that modulate IGF actions) are expressed early in CNS development (Baxter, 1991). So far six IGFBPs (IGFBP-1 through 6) have been identified. The IGFBPs have been suggested to augment the binding of IGF-I to its cell surface receptors. Binding sites for IGF-I have now been identified in virtually all brain regions of the adult rat brain using quantitative autoradiographic techniques (Bohannon *et al.*, 1988).

IGF-I is synthesized in the subventricular zone of the brain by type-1 astrocytes (Ballotti *et al.*, 1987; Bartlett *et al.*, 1991; Rotwein *et al.*, 1988). *In situ* hybridization studies have determined that, in the CNS of the rat, IGF-I expression begins by embryonic day 14 (Bartlett *et al.*, 1991) with peak expression of IGF-I occurring postnatally (Rotwein *et al.*, 1988; Werner *et al.*, 1989). The expression pattern of IGF system proteins during brain growth suggests highly regulated and developmentally timed IGF actions on specific populations of neurons. Apart from influencing neuronal development, IGF-I is also critical to oligodendroglial proliferation and differentiation.

In vitro, IGF-I has been shown to stimulate the proliferation of neuron progenitors and/or the survival of neurons and oligodendrocytes, and in some cultured neurons, to stimulate functions like neurite outgrowth and differentiation. McMorris *et al.* (1986) found that physiological concentrations of insulin and IGF-I induced the proliferation of O-2A progenitor cells. The proliferative effect of IGF-I on the O-2A progenitor cells was demonstrated by using tritiated thymidine incorporation of newly synthesized DNA as a marker of oligodendrocyte proliferation *in vitro* (McMorris and Dubois Dalcq, 1988). The same study also observed decreased tritiated thymidine labeling when cells were grown for two weeks or longer suggesting that IGF-I is not mitogenic to the O-2A lineage cells upon their differentiation into oligodendrocytes. It seems likely that both insulin and IGF-I together influence O-2A cell proliferation. This developmental feature gains indirect support from the evidence that both insulin (IR) and IGF receptor (IGFR) mRNAs have been found in O-2A progenitor cells (Han *et al.*, 1987; Shemer *et al.*, 1987; Van Schravendijk *et al.*, 1984). The insulin receptor mRNA levels found in O-2A progenitor cells is about 30 times more than that found in astrocytes or in neuronal cells (Baron Van Evercooren *et al.*, 1991). Accordingly, the facilitation of O-2A cell proliferation by insulin and IGF-I likely accounts for high proportion of O-2A cells found at day 12 in the *in vitro* model developed in the present thesis work.

Insulin and/or IGF-I act as a differentiation factor for the O-2A progenitor cells. The biological effects of insulin and IGF-I on oligodendroglial differentiation are triggered by the specific binding of these molecules to IR and IGFR (Mozell and McMorris, 1991). Receptor blocking experiments have revealed that the relative potency of induction of oligodendrocyte development depends on the binding affinity to IGFRs

(McMorris *et al.*, 1986). The beta subunit of both IR and IGFR consists of a transmembrane polypeptide chain, which contains a highly conserved catalytic tyrosine kinase domain. Within a few seconds of the binding of insulin and IGF-I to its receptors, the tyrosines undergo rapid phosphorylation. This subsequently leads to the phosphorylation of several intracellular substrates like Ras, Raf and phosphoinosityl- 3-kinase (PI-3 kinase) which eventually induce oligodendroglial proliferation and subsequent differentiation into the mature oligodendroglia.

The occurrence of IGF-I mRNA in both the gray and white matter areas of the developing rat brain indicated that developing oligodendrocytes might themselves be synthesizing IGF-I for their subsequent maturation (Rotwein *et al.*, 1988). This finding suggests that differentiation of oligodendrocyte precursors is not only regulated by the IGF-I produced by the type-1 astrocytes but also by the release of IGF-I in an autoregulatory manner. The influence of insulin and IGF-I on O-2A differentiation likely contributed to the rapid increase the number of PROLs and OLs between 12 and 16 DIV found in the present *in vitro* model.

1.3 Oligodendroglial Death

1.3.1 Developmental Death of O-2A Lineage Cells

Glial cells, particularly O-2A cells, are over produced during nervous system development. *In vivo* developmental studies have revealed that more than 50% of the newly formed O-2A progenitor cells undergo apoptosis (Barres *et al.*, 1992). The O-2A apoptosis has been seen to occur prior to differentiation and myelination of neuronal axons. The massive O-2A cell death has been hypothesized to occur in order to limit OL

numbers at levels that are appropriate to the axon surface areas requiring myelination. Cell death is not limited to the O-2A progenitor cell populations but also is seen the OLs. Recent studies have indicated that premyelinating OLs that go on to differentiate into myelinating OLs undergo cell death (Trapp *et al.*, 1997). Although the developmental cell death of OLs is now well recognized, it has not been determined whether or not it is apoptotic. If it is apoptotic, it is not known whether the process involves mitochondrial decisional points (see below) or whether it is responsive to anti-apoptotic compounds like (-)-deprenyl.

1.3.2 Oligodendrocyte Death in Pathological Conditions

OL death occurs not only during development but also in some pathological conditions. In human demyelinating diseases, like multiple sclerosis (MS), neurological manifestations depend on the axon populations that are involved by the demyelinating lesions and the functional deficits that result from impaired conduction in those axons. Neuropathological examination of the demyelinating MS foci has revealed a pronounced decrease in OL numbers, suggesting that the process involves OL death (Bruck *et al.*, 1994). Loss of OLs has been observed in both acute and chronic MS lesions.

The pathogenesis of MS has not been fully explained. The disease has been thought to result from a number of causes, principally aberrant autoimmunity directed against myelin antigens, possibly triggered by ubiquitous ribonucleic acid or deoxyribonucleic acid viruses (Scolding *et al.*, 1994; Simon and Neubert, 1996; Tienari, 1994). An inflammatory response accompanies the breakdown of myelin and the response is thought to be initiated by perivascular infiltration of $CD4^{+}$ T lymphocytes (Raine,

1994). It is believed that the activated T lymphocytes produce cytokines like lymphotoxin and tumor necrosis factor (TNF) which consequently act as major mediators of OL injury (Selmaj *et al.*, 1991; Selmaj and Raine, 1988). According to the view that an immune attack causes myelin damage and the consequences of the myelin damage result in OL damage, the decreased OL numbers in MS foci would be the result of OL death secondary to demyelination. Alternately though, immune or other damage to OLs could cause OL apoptosis with a resulting demyelination so that the decrease in OL numbers would be the primary rather than the secondary event in MS.

The decreased numbers of OLs in MS foci could also result from damage to the oligodendrocyte progenitor cells. Damage to O-2A progenitor cells could result from demyelinating periventricular lesions which are found in about 90% of MS brains (Lumsden, 1971). Periventricular lesions result in damage to the O-2A progenitor cells concentrated in the subependymal layer. The damaged oligodendrocyte progenitor cells would be unavailable to migrate from the subependymal layer and to replace OLs lost in the MS foci.

1.3.3 Oligodendrocytes and OL Progenitors Require Trophic Factors For Survival

Trophic factors have been considered above relative to their roles in influencing OL and type-2 astrocyte lineage differentiation. They also may play an important role in determining the survival of cells in the two lineages. There has been great interest in the role of trophic factors in the prevention of the death of neuronal cells (Thoenen, 1991). For example, trophic factors like CNTF, have been shown to ameliorate cell death of motoneurons *in vivo* when applied directly to the cells (Sendtner *et al.*, 1992) or into the cerebrospinal fluid (Zhang *et al.*, 1995). Other trophic factors, like nerve growth factor (NGF) prevents the death of several neuronal types like the sympathetic neurons and basal forebrain cholinergic neurons (Hefti, 1986; Oppenheim *et al.*, 1982).

Insulin, IGF-I, CNTF, PDGF and neurotrophins have been shown to increase the survival of OLs (Barres *et al.*, 1992a; Barres *et al.*, 1992b; Liu *et al.*, 1993; Louis *et al.*, 1993). The factors have also been shown to protect OLs from injury mediated by cytokines like TNF (D'Souza *et al.*, 1996; Louis *et al.*, 1993). Studies in the rat optic nerve have suggested that axonal activity modulates the release of the trophic factors (Barres *et al.*, 1993). Both *in vivo* and *in vitro*, astrocytes and neurons are considered to be the chief source of the growth and survival factors (see Henderson *et al.*, 1994; Seniuk-Tatton *et al.*, 1995) for evidence that both neurons and astrocytes synthesize CNTF).

1.3.4 Effects of Insulin, IGF-I and PDGF on Oligodendrocyte Survival

The importance of IGF-I in the survival of differentiated oligodendrocytes was suggested by the observation of hypermyelination in transgenic mice overexpressing IGF-I (Carson *et al.*, 1993). Other studies reported decreased numbers of oligodendrocytes in mice carrying homozygous disruptions of the IGF-I receptor (Liu *et al.*, 1993). Evidence for IGF-I regulation of normal myelination has also been obtained from nutritional studies (Wiggins, 1982). Those studies observed that nutritional deficiency during the postnatal developmental period in rats or mice resulted in decreased IGF-I levels and that the lowered IGF-I levels were associated with severe hypomyelination.

Studies using neuronal cell lines or primary cultures of neurons or OLs have indicated that not only IGFs' but also insulin regulates cell survival. Several *in vitro* studies have shown that high concentration of about 10μg / ml of insulin is essential for the survival of neuronal cells in serum free medium (Bottenstein, 1986; Eccleston and Silberberg, 1984; Saneto and de Vellis, 1985). The observation that supraphysiological concentrations of insulin enhanced the numbers of OLs *in vitro* suggested that insulin acted as an analogue of IGF-I by acting through the IGF-I receptors (McMorris, 1983; McMorris *et al.*, 1990).

Insulin and IGF-I appear to act cooperatively to regulate cell survival and death. Not much is known with respect to the role of insulin and IGF-I in regulating oligodendroglial survival, particularly whether their withdrawal leads to OL apoptosis. The present study established a culture model in order to investigate the capacity of insulin, IGF-I and serum withdrawal to initiate OL and type-2 astrocyte lineage apoptosis.

Other studies have utilized *in vitro* systems to examine the effects of trophic factors on the survival of oligodendroglial cells. *In vitro* studies have shown that exogenously added trophic factors or serum borne factors can influence oligodendroglial survival (Saneto and de Vellis, 1985; Raff *et al.*, 1983). Yonezawa and coworkers have shown that depletion of cystine from the media of oligodendroglial cultures caused an accumulation of intracellular free radicals resulting in oligodendroglial death (Yonezawa *et al.*, 1996). Barres *et al.* (1992) examined the survival of O-2A progenitor cells *in vitro*. Their study found that in the absence of serum and growth factors, the O-2A progenitor cells underwent rapid death according to a process, which included features suggestive of apoptosis. PDGF appeared to increase the O-2A progenitor cell survival. Barres and her colleagues performed experiments on O-2A progenitor cell survival in the intact mouse optic nerve. On transplantation of Cos-7 cells engineered to secrete PDGF in the subarachnoid space of the mouse brain, it was observed that in the intact mouse optic nerve, PDGF decreased O-2A cell death by about 85%. Furthermore, the numbers of surviving O-2A progenitor cells in the mouse optic nerve were seen to double over a period of four days.

Several studies have reported that the survival enhancing action of PDGF is specific to O-2A cells and not to OL cell populations (Hart *et al.*, 1989; McKinnon *et al.*, 1990). It is believed that lack of expression of PDGF receptors on differentiated oligodendroglial cells prevents PDGF from enhancing their survival. Therefore, these studies suggest that the survival of OLs is dependent on the presence of trophic factors other than PDGF, like insulin and IGF-I, yet prior to the experiments described in this thesis, there were no studies of the prevention of OL apoptosis by insulin and IGF-I.

1.4 Apoptotic Cell Death

Our understanding of apoptosis has advanced rapidly over the last several years. Nervous system apoptosis was thought to only result from trophic insufficiency. It is now known that apoptosis can be initiated in neural cells by many different forms of damage and proceeds in a step by step fashion with each step involving signaling by specific proteins. The signaling involves the cleavage, binding, phosphorylation or inter-organelle movements of proteins. The signaling pathways in the early phases of apoptosis depend on the form of damage that initiates the process. Accordingly, specific early pathways can be identified by changes in the levels and/or subcellular locations of specific proteins. The early signaling pathways converge onto a small number of effector signaling pathways that lead to the final degradative steps typical of apoptosis (see Susin *et al.*, 1996b).

In comparison to necrosis, apoptosis is a private kind of process. In necrosis, cells swell and their plasma membrane fracture allowing their intracellular contents to induce an inflammatory reaction. Inflammatory cytokines and other factors then damage and kill other nearby cells that were not involved in the primary process with the result that almost all cells in the region are killed. In contrast, apoptotic degradation is characterized by the formation of membrane wrapped fragments that do not induce an inflammatory reaction. The expression of surface markers on the wrapped fragments causes them to be engulfed by macrophages without damage to nearby cells. Accordingly, individual cells can die by apoptosis without involving their neighbors.

The individuality of apoptosis allows for the process to select specific cells for development without altering the overall organization of a neural structure (see Raff, 1992). It also allows apoptosis to serve as a selective defense system that can remove cells which are tumorigenic or infected by viruses (Vaux *et al.*, 1994) without compromising the function of a nervous system structure.

Nervous system apoptosis is not limited to development as it also occurs in pathological conditions (Chalmers-Redman *et al.*, 1996). The question of whether apoptosis mediates neuronal degeneration in neurological diseases such as Alzheimer's disease, Parkinson's disease, epilepsy, and ischemia/stroke is controversial. Participation of apoptosis in those conditions was largely missed on standard neuropathological examination due to the absence of inflammation and the selective culling of scattered cells. More recently development of fluorescent double labeling in situ methods used in conjunction with confocal laser imaging and deconvolution analysis have provided unequivocal evidence for the presence of apoptosis in those neurodegenerative conditions (Tatton and Kish, 1997; Tatton *et al.*, 1998).

As well as the evidence for apoptosis in MS presented above, it is now thought to contribute to Parkinson's disease (Mochizuki *et al.*, 1996; Tatton *et al.*, 1998; Olanow and Tatton, 1999), amyotrophic lateral sclerosis (Yoshiyama *et al.*, 1994), Huntington's disease (Thomas *et al.*, 1995) as well as acquired immunodeficiency syndrome (AIDS) (Petito and Roberts, 1995). In Alzheimer's disease, in particular, there is strong evidence from human brain studies, transgenic models and in vitro models to suggest that apoptosis contributes to nerve cell death (Anderson *et al.*, 1996; Su *et al.*, 1997; Dragunow *et al.*, 1997).

1.4.1 The Differentiation of Apoptosis and Necrosis

The occurrence of differences in the ultrastructure of cells dying by apoptosis or necrosis was first observed by Kerr who recognized a subpopulation of cells in the ischemic liver that were shrunken and retained intact lysosomal structure (Kerr, 1965). In contrast, necrotic hepatic cells were swollen with dissolution of lysosomes. Electron microscopy revealed that the shrunken cells had undergone cytoplasmic condensation and budding into membrane bound cell fragments, which were engulfed by phagocytes. Importantly, organelle structure was maintained within the cell fragments (Kerr, 1971).

In 1972, Kerr, Wyllie and Currie (Kerr *et al.*, 1972) detailed the ultrastructural features of the shrunken cells and termed the process as "apoptosis". They reported that the nuclei showed chromatin condensation and segregation into sharply delineated masses. They termed the condensed and fragmented cytoplasm that was membrane-wrapped as apoptotic bodies. The picture of apoptosis is slightly different *in vitro* where, in the absence of macrophages, apoptotic bodies persist for a longer period of time and may undergo cytolysis or secondary necrosis.

In 1980, Wyllie reported that the activation of endonucleases in apoptosis resulted in nuclear DNA fragmentation (Wyllie, 1980). Gel electrophoresis showed a 180-200 bp "ladder" pattern of DNA degradation indicative of internucleosomal DNA cleavage. DNA fragments of 300 or 500 bp in length are also formed in apoptosis and can be better detected with pulse field techniques (Sestili *et al.*, 1996). In contrast, in necrosis DNA cleavage is random producing a diffuse smear on DNA electrophoresis.

However, large amounts of tissue are required to detect DNA cleavage with these techniques, so they are not suitable for detecting small numbers of apoptotic nuclei and in a tissue with a number of different cell types, they do not allow the investigator to determine which types are apoptotic and which types are not. Nuclear DNA cleavage can be detected *in situ* using end labeling (ISEL) techniques, which attach a chromagen or fluorochrome to the 3' cut ends of DNA (Migheli *et al.*, 1994; Tatton and Kish, 1997). Chromatin condensation, which accompanies DNA fragmentation, can also be detected *in situ* staining with fluorescent DNA binding dyes such as acridine orange, Hoechst 33258 or YOYO-1 (Darzynkiewicz *et al.*, 1992; Tatton and Kish, 1997). Apoptosis can be unambiguously demonstrated if DNA ladder, ISEL positive nuclei and nuclear chromatin condensation are jointly demonstrated for the same cells.

1.4.2 Nerve Cell Apoptosis

It is now known that the massive death of neurons that occurs during mammalian prenatal and postnatal brain development depends on competition for trophic factors and represents a form of apoptosis (Martin *et al.*, 1992; Raff *et al.*, 1993). Nerve cells obtain their trophic support from their targets, which can supply limited amounts of trophic molecules. Limited supply of trophic molecules leads to a competition among the developing neurons so that neurons that are best connected survive while weakly connected neurons die (Oppenheim, 1991). The trophic insufficiency dependent death of developing neurons was termed "programmed cell death" since it depended on activation of a program of gene expression that led to the synthesis of "death proteins" (Oppenheim *et al.*, 1990).

A requirement for new protein synthesis can be a hallmark of programmed neuronal death or apoptosis, although it is not necessary for all forms of neuronal apoptosis (Dragunow and Preston, 1995; Johnson *et al.*, 1995). In some forms of neuronal apoptosis, proteins that are necessary for apoptosis to occur are constitutively expressed, while they must be newly synthesized in others (Eastman, 1993). The characteristic morphologic findings of apoptosis persist in many models of nerve cell apoptosis, even when transcriptional and translational blockers are used to inhibit new protein synthesis. For example, transcriptional or translational blockers that greatly reduce new protein synthesis do not prevent apoptosis after serum withdrawal in PC12 cells that have been exposed to serum but not to NGF (Rukenstein *et al.*, 1991). Similarly, PC12 cells that have been exposed to NGF for 6 days and have initiated process growth, undergo apoptosis after serum and NGF withdrawal that is independent of new protein synthesis (Tatton *et al.*, 1994). In contrast, apoptosis in neuronally-differentiated PC12 cells caused by trophic withdrawal after 9-12 days of NGF exposure requires new protein synthesis (Mesner *et al.*, 1992). Therefore, apoptosis can be dependent on new protein synthesis and be termed programmed cell death, or can be independent of new protein synthesis and be unprogrammed. Dependence on new protein synthesis can be used as a marker for neuronal apoptosis (Sanchez *et al.*, 1997; Ueda *et al.*, 1996) but absence of new protein synthesis does not rule out apoptosis (Deshpande *et al.*, 1992).

1.4.3 Changes in Gene Expression and Protein Levels in Apoptosis

In recent years, a number of genes and their protein products have been found to mediate the progression of apoptosis. Such genes and protein products can serve as

markers of apoptotic cell death process. Included among mediators of apoptosis are oncoproteins, oncogenes, and several protease families. The BCL-2 homolog family of oncoproteins includes both inhibitors and promoters of apoptosis (Hockenbery, 1992; Merry and Korsmeyer, 1997). Inhibitors of apoptosis include BCL-2 itself, BCL-X (BCL-X_L, BCL-Xα, and BCL-Xβ), MCL-1, and A1, while BAX, BCL-X_S, BAD, BAK, and BIK promote apoptosis. Some of the BCL-2 family members like BCL-2 and BAX can also interact with each other by forming homo- or heterodimers (Oltvai *et al*., 1993). Most of the oncoproteins are found localized to the outer mitochondrial and nuclear membranes. In normal hemopoetic cells, BCL-2 is concentrated in the outer mitochondrial membrane, while BAX is largely confined to the cytosol. However, during apoptosis BAX becomes concentrated in mitochondrial membranes (Hsu *et al*., 1997). Furthermore, in the early stages of apoptosis, a number of cytoplasmic proteins undergo nuclear translocation.

A number of proteases, including cysteine proteases, calpains, and proteasomes have been shown to be involved in the apoptotic cell death process. Most recent research efforts have been focused on the interleukin converting enzyme (ICE)-like proteases which are termed caspases (Cohen, 1997; Nath *et al*., 1996). To date, 10 different caspases have been identified with caspase-3 receiving most attention (Krajewska *et al*., 1997). The caspases are synthesized as inactive precursors and are activated in models of neuronal apoptosis (Armstrong *et al*., 1997; Du *et al*., 1997). Other proteases like calpains have been shown to contribute to the cleavage of the cytoskeletal protein actin during apoptosis (Brown *et al*., 1997). Calpain inhibitors have been shown to block actin cleavage in apoptosis (Gressner *et al*., 1997).

Cell-cycle related proteins and cycle kinases including cyclin D1, cyclin B, cyclin E, and Cdc2 kinase show increased levels during neuronal apoptosis (Freeman *et al.*, 1994; Gao and Zelenka, 1995; Haupt *et al.*, 1996; Kranenburg *et al.*, 1996). Appearance of the cycle-related proteins is thought to represent an abortive attempt of the post mitotic neuronal cells to return to the cell cycle (Shirvan *et al.*, 1997).

Another protein involved in the cell cycle as a mitosis inhibitor, p53, increases in a number of forms of neuronal apoptosis (Jordan *et al.*, 1997; Polyak *et al.*, 1997) and has been shown to increase the levels of the oncoprotein BAX in non-neuronal forms of apoptosis (Xiang *et al.*, 1998). It is now understood that apoptosis can be separated into p53 dependent and p53 independent forms (see Bellamy, 1997) for a detailed review of the role of p53 in apoptosis). Recently, a large number of p53-induced genes (PIGs) have been identified, which are proposed to mediate apoptosis by increasing oxidative radical levels, thereby inducing mitochondrial damage (Polyak *et al.*, 1997). p53-induced apoptosis can proceed after treatment with translational or transcriptional blockers (Kharlamov *et al.*, 1997), indicating that p53 can induce apoptosis through pathways that do not require new protein synthesis.

. A signal-transducing transcription factor of the AP-1 family, c-jun, is normally involved in cell cycle control, differentiation as well as regeneration. A variety of evidence indicates that c-JUN is involved in a number of *in vitro* and *in vivo* models of neuronal apoptosis (BossyWetzel *et al.*, 1997). Apoptosis is also induced by the Fas antigen and its mRNA has been shown to increase in ischemic nerve and glial cells (Depraetere and Golstein, 1997; Matsuyama *et al.*, 1995). Another marker for apoptosis is tissue transglutaminase, which cross-links cytoplasmic proteins and is increased in

apoptotic cells (Fesus *et al.*, 1991). Transglutaminase activity leads to the formation of high molecular mass protein polymers, which maintain the integrity of apoptotic cells and bodies and prevents leakage of their contents into the extracellular space.

Lastly, overexpression of the gene for the radical scavenger protein, Cu/Zn superoxide dismutase (SOD-1), reduces neuronal apoptosis (Greenlund *et al.*, 1995; Przedborski *et al.*, 1992) while underexpression increases apoptosis (Troy and Shelanski, 1994). Point mutations in the gene that encodes SOD-1 has been shown to convert the anti-apoptotic action of SOD-1 to a pro-apoptotic one (Rabizadeh *et al.*, 1995).

1.4.4 The Role of Mitochondria in Apoptosis

Mitochondria are now believed to play a critical role in signaling for the initiation of some forms of apoptosis. Apoptosis was initially believed to occur independently of mitochondrial factors (Jacobson *et al.*, 1993; Korsmeyer *et al.*, 1993). The importance of mitochondria in the initiation of apoptosis is illustrated by the finding that mitochondrial factors can induce chromatin condensation and nuclear fragmentation typical of apoptosis in cell free Xenopus egg extracts (Newmeyer *et al.*, 1994). Current evidence now indicates that apoptosis is associated with a sequence of events that includes a fall in mitochondrial membrane potential ($\Delta\Psi_M$), opening of a mitochondrial megapore known as the permeability transition pore (PTP), and release into the cytoplasm of small mitochondrial proteins which signal the initiation of apoptosis known as apoptosis initiating factors (AIF).

1.4.5 Mitochondrial Membrane Potential And Apoptosis

Studies of $\Delta\Psi_M$ have been pivotal in understanding the relationship between mitochondria and the initiation of apoptosis. An electrochemical proton gradient normally exists across the inner mitochondrial membrane resulting in a $\Delta\Psi_M$ of approximately -150 millivolts and a proton concentration difference (ΔpH) across the mitochondrial membrane. The $\Delta\Psi_M$ is dependent on the capacity of mitochondrial complexes I, III, and IV to use electron energy in the carrier molecules nicotinamide adenine dinucleotide (NADH), ubiquinone, and cytochrome C (CytC) to pump protons out of the mitochondrial matrix by transporting them across the inner mitochondrial membrane. Complex II transfers energy from $FADH_2$ to ubiquinone, but does not pump protons. The outward pumping of protons produces an electron gradient that is biochemically reflected by a pH difference (ΔpH) and electrically by a voltage across the inner mitochondrial membrane also known as $\Delta\Psi_M$ (Sheratt, 1991). The $\Delta\Psi_M$ and the ΔpH contribute to a proton electromotive force (δp) ($\delta p = \Delta\Psi - 60\ \Delta pH$, where ΔpH = mitochondrial pH - cytosol pH). δp drives the conversion of ADP to ATP at complex V (ATP synthase). Since $\Delta\Psi_M$ is by far the greater contributor to δp, $\Delta\Psi_M$ can be assumed to vary almost linearly with the ATP/ADP ratio and to provide an estimate of the ATP/ADP ratio within individual mitochondria.

Measurements of whole cell potentiometric dye fluorescence in a variety of blood, hepatic, and immune cell models have shown that $\Delta\Psi_M$ is reduced very early in the apoptotic process, prior to the onset of nuclear DNA fragmentation and chromatin

condensation (Susin *et al.*, 1996b). More recently, Tatton and coworkers used laser confocal imaging to obtain direct measurements of $\Delta\Psi_M$ in living NGF-differentiated PC12 cells and showed that a decrease in $\Delta\Psi_M$ is also one of the earliest, if not the earliest, detectable event in apoptosis induced by NGF and serum withdrawal (Wadia *et al.*, 1998). $\Delta\Psi_M$ was significantly reduced in a proportion of mitochondria three to six hours prior to nuclear DNA fragmentation and chromatin condensation. The decrease in $\Delta\Psi_M$ was temporally correlated with an increase in intramitochondrial Ca^{2+} but not with an increase in cytosolic oxidative radical levels, which increased only after the decrease in $\Delta\Psi_M$ was well established. Decreases in $\Delta\Psi_M$ coupled with increases in intramitochondrial Ca^{2+} induce opening of the PTP (see below (Scorrano *et al.*, 1997). Accordingly, these changes found in early apoptosis were appropriate to open the PTP.

1.4.6 The Permeability Transition Pore

A decrease in $\Delta\Psi_M$, in the presence of increased intramitochondrial Ca^{2+} (Scorrano *et al.*, 1997), induces opening of a PTP, which spans the inner and outer mitochondrial membranes. The PTP consists of an adenine nucleotide translocator (AdNT) which is a critical element of the PTP but whether it forms the pore itself or is just closely associated with a pore forming protein is unknown. The AdNT has been specifically thought to be associated with the PTP because of the observation that ligands of AdNT like atractyloside, bongkrekic acid and ADP affect the PTP by acting as its inducers to open the PTP (Zoratti and Szabo, 1995). The PTP also includes a voltage dependent anion channel (a porin) and a peripheral benzodiazepine binding protein. It is now believed that two porin molecules of 2-3 nm diameter may cooperatively form the

anion channel. The components of the PTP are closely associated with hexokinase, creatine kinase, and BCL-2, as well as other elements, such as glycerol kinase, phospholipid hydroperoxidase, glutathione peroxidase, 3-b-hydroxysteroid dehydrogenase isomerase, and cardiolipin synthase. Recent studies have suggested complex protein-protein interactions of the elements of the PTP (Zamzami et al., 1998). Direct interactions have been suggested to occur between AdNT and porin as well as between hexokinase and porin. Other protein-protein interactions within the PTP remain to be elucidated.

The PTP opens in response to a decrease in $\Delta\Psi_M$ in the presence of an increase in intramitochondrial Ca^{2+} (Scorrano *et al.*, 1997). Marked increases in mitochondrial Ca^{2+}, increased oxidative radical levels, or partial failure of the respiratory complexes, acting either individually or together, can induce a fall in $\Delta\Psi_M$ (Richter, 1993). Opening of the PTP dissipates any remaining proton gradient across the mitochondrial membrane and further reduces the $\Delta\Psi_M$ (Bernardi *et al.*, 1994). Complete opening of the PTP allows free exchange of solutes and small proteins between the mitochondrial matrix and the extramitochondrial cytosol. Consequently mitochondria swell rupturing the outer mitochondrial membrane. As a result AIFs are released from the intermembrane space of the mitochondria into the cytoplasm (Marchetti *et al.*, 1996; Susin *et al.*, 1996b). Mitochondrial AIFs may be released directly through the PTP or through fractures in the mitochondrial membrane.

Several factors are known to influence opening or closure of the PTP. Compounds like cyclosporin A bind to the PTP and maintains it in a closed position. It also promotes pore closure by binding cyclophilins that otherwise induce PTP opening in the presence

of Ca^{2+} by binding to the AdNT (Bernardi *et al.*, 1994; Scorrano *et al.*, 1997). Factors like glutathione, ADP levels, and ROS levels in the mitochondrial matrix modulate the gating voltages necessary to induce PTP opening cooperatively by oxidation-reduction events at two separate sites of the PTP but are not sufficient in themselves to open the PTP (Chernyak and Bernardi, 1996).

Anti-apoptotic proteins like BCL-2 bind to the PTP. BCL-2, binds to the peripheral benzodiazepine binding component of the mitochondrial PTP (Carayon *et al.*, 1996) and maintains closure of the PTP in a manner similar to cyclosporin A. BCL-2 has been shown to localize to the outer mitochondrial membrane (Lithgow *et al.*, 1994; Riparbelli *et al.*, 1995) within or near to, the mitochondrial peripheral benzodiazepine receptor (Carayon *et al.*, 1996). Cytosolic BCL-2, which is truncated and cannot dock in mitochondrial membranes, is considerably less effective in reducing apoptosis than BCL-2 located in mitochondrial membranes (Hockenbery *et al.*, 1993). Richter first proposed that BCL-2 reduces apoptosis by maintaining $\Delta\Psi_M$ (Richter, 1993). Zamzami and coworkers subsequently provided persuasive evidence, demonstrating that BCL-2 maintains PTP closure (Zamzami *et al.*, 1996) and blocks the initiation of apoptosis by preventing the escape of heat labile molecules, which signal the onset of apoptosis. Numerous other studies have also shown that BCL-2 can prevent a decrease in $\Delta\Psi_M$ and the release of ICE-like AIFs (Marchetti *et al.*, 1996; Susin *et al.*, 1996b). Recently. Marzo and coworkers have shown that the proapoptotic protein, Bax binds to AdNT of the PTP, which results in the opening of the PTP with resultant apoptotic death (Marzo *et al.*, 1998). Thus, opening of the PTP can be viewed as the critical decisional step in many forms of apoptosis and is proposed to constitute an irreversible step in the process.

1.4.7 Apoptosis Initiating Factors

The importance of mitochondrial factors in the initiation of apoptosis has been demonstrated by studies showing that mitochondrial homogenates are essential for the progression of the nuclear changes of apoptosis in cell free systems (Newmeyer *et al.*, 1994). Several apoptosis promoting mitochondrial AIFs have been identified to date. dATP, when accompanied by holocytochrome C, a nuclearly encoded 14 kDa protein, which is normally localized to the mitochondrial intermembranous space, can promote apoptosis in some cell free systems (Liu *et al.*, 1996). Additionally, in some forms of apoptosis, CytC can be found in the extramitochondrial cytosol in the early stages of apoptosis (Yang *et al.*, 1997) and injection of CytC into cells induces apoptosis (Li *et al.*, 1997). CytC release from mitochondria has been shown to activate a caspase 3 precursor leading to activation of an endonuclease that cleaves nuclear DNA (Kharbanda *et al.*, 1997; Li *et al.*, 1997).

1.4.8 Oxidant Radicals and Mitochondrially Induced Apoptosis

An increase in oxidative radicals derived from the mitochondria is thought to be associated with apoptosis (Richter *et al.*, 1995). Increased levels of oxidative radicals, particularly in the presence of increased intramitochondrial Ca^{2+}, can induce apoptosis by causing cross linking of protein thiols in the mitochondrial inner membrane (van de Water *et al.*, 1994) and opening of the PTP (Chernyak and Bernardi, 1996). BCL-2 which has been shown to act as an anti-oxidant is thought to provide anti-apoptotic effects through an indirect effect on the PTP as well as by its direct effect on the PTP

(Hockenbery *et al.*, 1993). Tatton and his co-workers have shown that a decrease in BCL-2 levels in trophically-deprived PC12 cells entering apoptosis is associated with markedly increased cytosolic levels of oxidative radicals (Tatton *et al.*, 1996). In contrast, the reduction in apoptosis induced by bcl-2 overexpression is associated with a decrease in both oxidative radical levels and in peroxidation of membrane lipids (Hockenbery *et al.*, 1993). Similar effects have been detected with oxidative radical scavengers, such as SOD-1 and glutathione, which have been shown to prevent the direct action of oxidative radicals on PTP opening (Chernyak and Bernardi, 1996). These direct and indirect actions of oxidative radicals on the PTP may reinforce each other in inducing apoptosis.

1.5 (-) -Deprenyl Can Reduce Apoptosis

(-) -Deprenyl is a selective inhibitor of monoamine oxidase B (MAO-B) (Heinonen *et al.*, 1994). Co-administration of (-) -deprenyl or other MAO-B inhibitors with the parkinsonian toxin, 1-methyl-4-phenyl -1,2,3,6-tetrahydropyridine (MPTP) reduced the decreases in striatal dopaminergic indices caused by the toxin alone (Cohen *et al.*, 1984; Heikkila *et al.*, 1984; Langston *et al.*, 1984). The reduction was interpreted to depend on the blockade of the conversion of MPTP to its active radical, MPP^+ , by MAO-B in astroglia. If MPTP could not be converted to MPP^+, then it could not induce necrosis of substantia nigra dopaminergic neurons (SNDns). Necrosis of SNDns results in the loss of their striatal dopaminergic terminal axons and a decrease in stiatal dopaminergic indices. Accordingly, the maintenance of the striatal dopaminergic indices was interpreted as showing that (-) -deprenyl reduced the necrosis of SNDns caused by MPP^+.

Subsequently, it was found: 1) that (-) -deprenyl reduced the death of cultured dopaminergic neurons caused by MPP^+ (Mytilineou and Cohen, 1985), 2) that (-) -deprenyl reduced the loss of murine SNDns when (-) -deprenyl treatment was delayed until after the conversion of MPTP to MPP+ was completed (Tatton and Greenwood, 1991) or when (-) -deprenyl doses were employed that were insufficient to inhibit MAO-B or MAO-A (Tatton *et al.*, 1993) and 3) that (-) -deprenyl could increase the survival of non-dopaminergic neurons and axotomized motoneurons, independently of MAO inhibition (Ansari *et al.*, 1993; Salo and Tatton, 1992). Those studies established that (-) -deprenyl could increase neuronal survival through a mechanism that did not require the

inhibition of MAO-B.

Studies in trophically-withdrawn neuronally differentiated PC12 cells (Tatton *et al.*, 1994; Wadia *et al.*, 1998), hippocampal pyramidal cells subjected to ischemia (Paterson *et al.*, 1997), cerebellar granule cells exposed to cytosine arabinoside (Paterson *et al.*, 1998), trophically deprived human melanoma cells (Magyar *et al.*, 1998), serum deprived immortalized rat neural retinal ganglion cells (Ragaiey *et al.*, 1997), rotenone exposed human neuroblastoma PAJU cell line (Kragten *et al.*, 1998), peroxynitrite and nitric oxide exposed human dopaminergic neuroblastoma SH-SY5Y cells (Maruyama *et al.*, 1998) and dopaminergic MES23.5 cells exposed to MPP+ (Le *et al.*, 1997) showed that (-)-deprenyl can reduce apoptotic cell death. Since the original reports of MAO-B independent neuronal rescue, more than thirty studies have reported reduced nerve cell death by (-)-deprenyl for a variety of different types of neurons including motoneurons (Ansari *et al.*, 1993; Iwasaki *et al.*, 1996; Ju *et al.*, 1994; Oh *et al.*, 1994; Ravikumar *et al.*, 1998; Salo and Tatton, 1992; Zhang *et al.*, 1995), neuronally differentiated PC12 cells (Tatton *et al.*, 1994), retinal cells (Buys, 1995), inferior olivary neurons (Todd and Butterworth, 1998), inferior collicular neurons (Todd and Butterworth, 1998), thalamic neurons (Todd and Butterworth, 1998), mesencephalic dopaminergic neurons (Koutsilieri *et al.*, 1996; Koutsilieri *et al.*, 1994; Mytilineou *et al.*, 1997; Wu *et al.*, 1995), cerebellar neurons (Paterson *et al.*, 1998), cortical neurons (Amenta *et al.*, 1994), striatal neurons (Vizuete *et al.*, 1993) and hippocampal neurons (Amenta *et al.*, 1994; Knollema *et al.*, 1995; Lahtinen *et al.*, 1997; Paterson *et al.*, 1997; Semkova *et al.*, 1996; Zeng *et al.*, 1995; Zhang *et al.*, 1996). Increased neuronal survival has been found after a wide variety of different insults including trophic withdrawal (Tatton *et al.*, 1994), MPP^{+}

exposure (Koutsilieri *et al.*, 1996; Koutsilieri *et al.*, 1994; Schmidt *et al.*, 1997; Wu *et al.*, 1995) , excitotoxicity (Abakumova *et al.*, 1998; Kiran *et al.*, 1998; Mytilineou *et al.*, 1997; Semkova *et al.*, 1996), ischemia or hypoxia (Knollema *et al.*, 1995; Lahtinen *et al.*, 1997; Paterson *et al.*, 1997; Ravikumar *et al.*, 1998), DNA toxins (Paterson *et al.*, 1998), axotomy (Ansari *et al.*, 1993; Buys, 1995; Ju *et al.*, 1994; Oh *et al.*, 1994; Salo and Tatton, 1992; Zhang *et al.*, 1995), thiamine deficiency (Todd and Butterworth, 1998), and catecholaminergic toxins (Zhang *et al.*, 1996).

(-)-Deprenyl has not been universally effective in reducing cell death (Ballabriga *et al.*, 1997; Fang *et al.*, 1995; Rothblat and Schneider, 1998; Thiffault *et al.*, 1997; Vaglini *et al.*, 1996). In some cases, (-) -deprenyl has been found to be highly effective or ineffective in reducing neuronal death in similar models. For example, three groups exposed adult gerbils to 5 minutes of bilateral carotid occlusion and began daily (-) -deprenyl treatment at 1-2 hours after the occlusion. One group evaluated CA1 hippocampal neuronal numbers at day 4 after daily treatment with 10 mg/kg (-) -deprenyl and found no increase in survival (Ballabriga *et al.*, 1997). The other evaluated CA1 hippocampal neuronal numbers at days 3 or 7 after treatment with 0.25 mg/kg and found a significant increase in survival at both time points (Lahtinen *et al.*, 1997). A third group evaluated 0.25 mg/kg daily (-)-deprenyl treatment on hypoxia/ischemia induced rat CA1, CA3 and CA4 hippocampal neuronal death at multiple time points up to 14 days and found markedly significant increases in neuronal survival (Paterson *et al.*, 1997).

Three different factors have been proposed to account for models in which (-)-deprenyl is ineffective: 1) that (-) -deprenyl can only reduce neuronal death mediated by

apoptosis but not that mediated by necrosis. For example high levels of MPTP or MPP+ induce necrosis while lower levels induce apoptosis (Hartley *et al.*, 1994; Mochizuki *et al.*, 1994; Spooren *et al.*, 1998; Tatton *et al.*, 1997). The levels of MPTP or MPP+ that were employed may explain studies in which (-) -deprenyl was effective (Koutsilieri *et al.*, 1996; Koutsilieri *et al.*, 1994; Mytilineou and Cohen, 1985; Schmidt *et al.*, 1997; Tatton and Greenwood, 1991; Tatton *et al.*, 1993; Wu *et al.*, 1995) versus those in which it was ineffective (Rothblat and Schneider, 1998; Thiffault *et al.*, 1997; Vaglini *et al.*, 1996). Similar variability in the rapidity and depth of hypoxia may account for the variable effectiveness of (-)-deprenyl in hypoxia/ischemia models considered above. 2) that (-)-deprenyl may only by effective in some forms of apoptosis but not in other forms. Paterson showed that (-)-deprenyl reduced apoptosis of cultured cerebellar granule cells induced by cytosine arabinoside but not that induced by low potassium levels (Paterson *et al.*, 1998). Similarly our group has found that (-)-deprenyl reduces apoptosis in PC12 cells that have been exposed to serum and NGF (Tatton *et al.*, 1994; Wadia *et al.*, 1998) but not those that have only been exposed to serum (Chalmers-Redman and Tatton , unpublished observations). Similarly, (-)-deprenyl does not reduce apoptosis in the classic model of dexamethasone-exposed lymphocytes (Fang *et al.*, 1995). Paterson *et al.* (1998) proposed that (-)-deprenyl only reduces those forms of apoptosis that involve a mitochondrial decision point (see above). For example, cytosine arabinoside induced apoptosis of cerebellar granule cells involves an early decrease in $\Delta\Psi_M$ while apoptosis induced by low potassium in the same cells does not involve early decreases in $\Delta\Psi_M$. (-)-Deprenyl is effective in the former but not the latter. Similarly,

apoptosis in PC12 cells exposed to serum and NGF involves an early decrease in $\Delta\Psi_M$ and is responsive to (-)-deprenyl (Wadia *et al.*, 1998) while that in PC12 cells that have only been exposed to serum does not involve an early decrease in $\Delta\Psi_M$ and is unresponsive to (-)-deprenyl (Chalmers-Redman and Tatton , unpublished observations). Lastly, dexamethasone induced lymphocyte apoptosis does not involve an early decrease in $\Delta\Psi_M$ (Zamzami, personal communication) and is not (-)-deprenyl responsive. Accordingly, those forms of apoptosis that do involve an early decrease in $\Delta\Psi_M$ may not respond to (-)-deprenyl. 3) Recently, it has been suggested, but not proven, that the primary metabolite of (-)-deprenyl, (-)-desmethyldeprenyl (DES), mediates anti-apoptosis rather than (-)-deprenyl itself (Mytilineou *et al.*, 1997). Accordingly, variations in (-)-deprenyl effectiveness from model to model may depend on the tissues capacity to convert (-)-deprenyl to DES.

1.5.1 (-)-Deprenyl Requires New Protein Synthesis to Reduce Apoptosis and Alters Gene Expression and Protein Synthesis in Pre-Apoptotic Cells

Studies in neuronally-differentiated PC12 cells showed that transcriptional inhibition by actinomycin and translational inhibition by cycloheximide or campothecin blocked the capacity of (-)-deprenyl to reduce apoptosis in the cells (Tatton *et al.*, 1994). In a variety of cell types, (-)-deprenyl has been shown to increase gene expression, protein levels or protein activity for superoxide dismutases (SOD1 and SOD2) (Carrillo *et al.*, 1991; Kitani *et al.*, 1994; Li *et al.*, 1998; Thiffault *et al.*, 1995), BCL-2 (Tatton and Chalmers Redman, 1996), heat shock protein 70 (Lahtinen *et al.*, 1997; Zhang *et al.*, 1996), GFAP (Amenta *et al.*, 1994; Biagini *et al.*, 1994; Biagini *et*

al., 1993; Ju *et al.*, 1994; Li *et al.*, 1993; Revuelta *et al.*, 1997), aromatic amino acid decarboxylase (Li *et al.*, 1992), tyrosine hydroxylase (Li *et al.*, 1997; Reinhard and JP, 1991; Rodriguez-Gomez *et al.*, 1997), CNTF (Seniuk *et al.*, 1994), NGF (Li *et al.*, 1997; Semkova *et al.*, 1996), FGF (Biagini *et al.*, 1994), and trk receptor mRNA (Ekblom *et al.*, 1994).

It is not known whether any of the above proteins are associated with the capacity of (-)-deprenyl to reduce apoptosis. SOD1underexpression increases apoptosis while its overexpression decreases apoptosis in PC12 cells and sympathetoblasts (Greenlund *et al.*, 1995; Troy and Shelanski, 1994) making an increase in SOD1 expression a candidate for the anti-apoptotic effects of (-)-deprenyl. The capacity of (-)-deprenyl to increase SOD1 has been shown to vary from tissue to tissue (Carrillo *et al.*, 1992; Lai *et al.*, 1994), which might also explain the variability in (-)-deprenyl effectiveness. BCL-2 may also contribute to (-)-deprenyl anti-apoptosis. BCL-2 has been shown to prevent the fall of $\Delta\Psi_M$ that is characteristic of some forms of apoptosis (Kroemer *et al.*, 1997; Susin *et al.*, 1996a) and (-)-deprenyl also prevents a fall in $\Delta\Psi_M$ in neuronally differentiated PC12 cells (Wadia *et al.*, 1998) and cerebellar granule cell apoptosis (Paterson *et al.*, 1998). A decrease in BCL-2 levels has been shown to contribute to the fall in $\Delta\Psi_M$ in apoptotic cells (Kroemer *et al.*, 1997; Susin *et al.*, 1996a) while a maintenance of BCL-2 through its new synthesis would prevent a fall in $\Delta\Psi_M$ and the initiation of apoptosis.

It has been suggested that the anti-apoptotic action of (-)-deprenyl on neurons is mediated by the capacity of the compound to increase the synthesis of CNTF (Seniuk *et al.*, 1994), NGF (Semkova *et al.*, 1996) and bFGF (Biagini *et al.*, 1994) by astrocytes (Koutsilieri *et al.*, 1996). According to that proposal, (-)-deprenyl would increase the

provision of trophic support to damaged neurons on astroglia and thereby reduce apoptosis.

It is not known whether (-)-deprenyl or one or more of its metabolites can influence apoptosis in oligodendroglial cells. Furthermore, it is not known whether any effects of (-)-deprenyl on oligodendroglial cells involves new protein synthesis and/or whether (-)-deprenyl has any effect on $\Delta\Psi_M$ in the cells.

1.6 Specific Aims

The specific experimental aims of this thesis were:

1. To establish a tissue culture model system to study whether insulin, IGF-I and serum withdrawal causes apoptosis in differentiated cells of the O-2A cell lineage like the PROLs and OLs similar to that observed in the progenitor cell population. For this study, I have established primary cultures of oligodendrocytes by obtaining purified populations of O-2A progenitor cells isolated from the rat cerebral cortex and differentiating them in the presence of trophic factors, insulin and IGF-I. The different cell types of the O-2A lineage were then identified on the basis of morphological and immunocytochemical criteria. Apoptotic degradation was studied using multiple complementary approaches including a) staining of chromatin with fluorescent DNA binding dyes; b) gel electrophoresis of DNA and c) *in situ* end labeling (ISEL) of nicked 3' ends of DNA.
2. To determine the time course of any apoptotic death of the insulin, IGF-I and serum withdrawn PROLs and OLs by obtaining counts of ISEL positive apoptotic nuclei of PROLs and OLs after 6, 12, 15, 18 and 24 h after withdrawal of insulin and IGF-I.
3. To determine whether (-) -deprenyl can reduce apoptosis in PROLs and OLs by

obtaining nuclear and cell counts. The numbers of ISEL positive nuclei were also obtained to determine whether they are appropriately reciprocal to the cell numbers. DNA electrophoresis gels were used to determine whether (-)-deprenyl eliminates or reduces DNA "laddering" at appropriate time points. Additionally Western blot analysis was used to determine whether (-)-deprenyl modified the levels of OL specific proteins similar to the changes seen in cell numbers.

4. To determine whether any anti-apoptotic effects on the PROLs and the OLs are brought about by (-) -deprenyl itself or by one of its metabolites, like (-)-desmethyldeprenyl. The intact nuclear count method was used to quantify the effects of the metabolites of (-) -deprenyl on the survival of the PROLs and the OLs. Three general cytochrome P450 blockers were used to determine if the blocking of metabolism of (-)-deprenyl or a metabolite eliminates any increased PROL and OL survival induced by the compound.

5. To determine whether any anti-apoptotic action of (-)-deprenyl and /or a major metabolite requires new protein synthesis by using actinomycin D to inhibit transcription and cycloheximide to inhibit translation.

6. To determine whether a decrease in $\Delta\Psi_M$ is involved in any OL and PROL apoptosis induced by insulin, IGF-I and serum withdrawal. The involvement of mitochondria in the apoptotic death of PROLs and OLs was determined using $\Delta\Psi_M$ changes as an indicator. I have used the mitochondrial potentiometric dye, chloromethyltetramethyl rhodamine (CMTMR) which was imaged in individual mitochondria in intact cells using confocal laser microscopy.

7. To determine whether treatments with (-)-deprenyl or a major metabolite or those

that block decreases in any apoptosis in PROLs and OLs have appropriate actions on $\Delta\Psi_M$.

2.0 Materials and Methods

2.1.1 Preparation of Mixed Primary Glial Cultures

Primary cultures enriched in O-2A progenitors grown over a monolayer of astrocytes were derived from the cerebral cortex of 2-day-old rat pups. Timed pregnant Sprague Dawley rats (Charles River, Montreal, Canada) were obtained at 18 days gestation and housed in the Carleton Animal Care Facility at Dalhousie University. Two litters (consisting of about 10-12 pups / litter), obtained at postnatal day 2, were used in each experiment. The rat pups were anesthetized with a brief exposure to an inhalant anesthetic, Metofane and then quickly decapitated. Using sterile technique, the skull was opened via the foramen magnum towards the olfactory bulb and the skull flaps were pried open carefully. With a microspatula, the entire brain was lifted away and placed in cold Dulbecco's modified Eagle's medium (DMEM; GibcoBRL, Life Technologies, Ontario, Canada) supplemented with 15% fetal bovine serum (FBS; Hyclone Laboratories Inc, Utah, USA). Subsequently, the meninges were removed and the cerebral hemispheres transferred to another sterile culture dish. The cerebral hemispheres were then dissected under a stereomicroscope and the cortices isolated free of the midbrain, hippocampus and the striatum. Using a cell scraper all the neocortical tissue was mechanically dissociated and filtered successively through 210 and 130 μm Nitex meshes (B&SH Thompson & Co Ltd, Ontario, Canada). This procedure allows for the elimination of contaminating fibroblasts. The cell suspension was centrifuged at 300 x g in 50 ml tubes using a Sanyo Mistral 3000i centrifuge and the cells pelleted and resuspended in DMEM containing 15% serum. Viable cells, as determined by the Trypan Blue dye exclusion, were counted

on a haemocytometer and plated at a density of 1.37×10^6 cells / ml onto 185 cm^2 Nunc culture flasks.

The mixed cultures were grown for 10 days in a media containing DMEM, 15% FBS, penicillin (50 IU / ml), streptomycin (50 μg / ml), 1mM sodium pyruvate; 50 μg / ml transferrin (Sigma Chemical Co., MO, USA) ; 0.005 μg / ml sodium selenite (Sigma) and 5 μg / ml insulin (Upstate Biotechnology Inc, NY, USA) until confluent. The media was replaced every 3 days. Most neurons in the culture fail to survive and are eliminated by the second change of the media.

2.1.2 Preparation of Secondary Cultures

Secondary cultures enriched in oligodendrocytes were obtained by a modification of the selective adhesion protocol of (McCarthy, 1980). By 10 days *in vitro* (DIV), the O-2A progenitor cells had spread in clusters over the confluent layer of astrocytes. The flasks were then subjected to a 'preshake' at 170 rpm for 1 hour, 37°C, (in order to release macrophages, dead cells and debris) on an orbital shaker (Aros 160, Thermolyne) with an inch and a quarter stroke diameter. The preshake was followed by an 18 hour shake at 230 rpm. Medium containing the detached O-2A cells was recovered and filtered through a 20 μm Nitex mesh to remove clumps of dead cells and larger type-1 astrocytes. The cell filtrate was plated for 5 min in a plastic petridish in order to separate the O-2A progenitor cells from microglia. The loosely adherent O-2A cells were detached by incubating them in a solution of 10 mM Tris and 1 mM EDTA (pH 7.4) at 37°C for 5 min. The cells were subjected to centrifugation at 300 x g for 5 min and cells were resuspended in an oligodendrocyte differentiating media (ODM) containing DMEM,

10% FBS, 16 μg / ml putrescine (Sigma), 0.006 μg / ml progesterone (Sigma), 5 μg / ml insulin, 2.5 ng / ml insulin-like growth factor-1 (Upstate), 50 μg / ml transferrin and 0.005 μg / ml sodium selenite. The ODM was based on a modification of the defined medium as described by Hunter (Hunter and Bottenstein, 1990).

The cell suspension was plated on 12 mm and 22-mm coverslips (Assistent Glass, Carolina Biological Supply Co., Burlington, NC, USA) as well as 24 well Nunc plastic plates. The coverslips used were alkaline etched (NaOH, 0.5 N) for 30 minutes. The coverslips were then thoroughly washed with distilled water, autoclaved and subsequently poly-L-lysine (Sigma, P6282) coated. The cell suspension was plated at two different densities: 1) 5.3×10^3 cells / mm^2 (experimental series I) and 2) 15.8×10^3 cells / mm^2 (experimental series II). Two separate experiments constituted each experimental series. The cells were maintained in ODM with 10% FBS for 3 hr at 37°C to enhance survival following which the media was replaced with ODM with low serum (1% FBS). The O-2A progenitors were then allowed to differentiate for a further 6 days, and monitored by phase contrast microscopy to determine when the majority of the cells could be morphologically identified as OLs. Phase contrast micrographs of the differentiating O-2A progenitors were taken using a Leitz inverted phase contrast microscope coupled to a charge coupling device (CCD) camera.

2.2 Immunocytochemical Detection of Cultured Oligodendroglial Cells

Cells growing on coverslips were identified by immunocytochemistry for specific markers of oligodendrocyte development like A2B5 (Eisenbarth *et al.*, 1979), 2'-3'-cyclicnucleotide - 3'- phosphorylase (CNPase) (Knapp, 1988), galactocerebroside (GC) (Ranscht, 1982), myelin basic protein (MBP) (Lees, 1984) and proteolipid protein (PLP) (Bartlett *et al.*, 1988).

Cells growing on coverslips were fixed with 2% paraformaldehyde for 15 minutes at room temperature. The coverslips were then washed three times with 0.1M phosphate buffered saline (PBS). Non-specific binding of protein was blocked by incubating the coverslips in 10% normal goat serum at room temperature for an hour, followed by permeabilization with 0.1% Tween-20 (Sigma) for 20 minutes at room temperature. Endogenous peroxidase was blocked by incubating the coverslips in 1% H_2O_2 for 10 minutes. Subsequently, the coverslips were incubated overnight at 4°C with primary antibodies. PROLs were identified by a using a primary antibody, mouse anti CNPase (1:100; Sigma C-5922) while OLs were identified using antibodies, rabbit anti MBP (1:100; Zymed Laboratories Inc, San Francisco, CA, USA #18-0038) and rat anti PLP 1:100; Immunodiagnostics Inc, Bedford, MA, USA #9021). Astrocytes were identified by mouse anti glial fibrillary acidic protein (GFAP) (1:200; Sigma G-3893). All primary antibodies were diluted in 0.1M PBS / 1% normal goat serum / 0.1% Tween-20. In order to detect surface antigens present in O-2A progenitors (A2B5) and PROLs (GC), antibodies mouse anti A2B5 (1:100; Boehringer Mannheim, Quebec, Canada, 1300 016), rabbit anti-GC (1:50; Boehringer Mannheim, 1351 621) were applied to living cells for

30 minutes.

Following the primary antibody incubation, coverslips were washed 3 times with 0.1M PBS. Coverslips were then incubated with the appropriate biotinylated goat anti-mouse, goat anti-rabbit or goat anti-rat IgG secondary antibody (1:500; Vector Labs) at room temperature for one hour. Subsequently, after three washes with 0.1M PBS the coverslips were incubated for 30 minutes with avidin-HRP (Elite Kit, Dimension Laboratories Inc., Missisauga, Ontario, Canada). Coverslips were washed with PBS and chromogenic detection was carried out using diaminobenzidine (DAB, 1.5 mg / ml) mixed with an equal volume of hydrogen peroxide (H_2O_2; 0.02% v/v) to obtain a brown reaction product within 5 min localizing the specific antigens. Coverslips were then dehydrated in ascending grade alcohol series, cleared with xylene and mounted on slides using a drop of permount. Controls for immunocytochemical staining for each antibody used in the present study were run in parallel by substituting the primary antibody with PBS.

2.3 Insulin, IGF-I and Serum Withdrawal and Culture Treatment Conditions

In order to examine insulin and IGF-I dependent survival of the cells of the oligodendroglial lineage, cells were trophically deprived for a period of 24 hours at 16 DIV. At this time in culture, the O-2A progenitor cells had differentiated into CNPase immunopositive PROLs and MBP immunopositive OLs.

Cells were trophically withdrawn by replacing the media with a serum free, chemically-defined base media (BM) lacking insulin and IGF-I (Bottenstein *et al.*, 1988). The base media consisted of DMEM supplemented with penicillin (50 IU / ml);

streptomycin (50 μg / ml); 1mM sodium pyruvate; 50 μg / ml transferrin; 0.005 μg / ml sodium selenite; 16 μg / ml putrescine; 0.006 μg / ml progesterone and 0.1 mg / ml bovine serum albumin (99%purity; Sigma). All the cells were washed with BM twice before exposing them to individual treatment conditions. Control cells were washed and placed in BM supplemented with 2.5 ng / ml IGF-I and 5 μg / ml insulin to form the insulin media (IM).

To study the effects of trophic-like agents on the survival of oligodendroglial lineage cells, cultures were exposed to varying concentrations of (-)-deprenyl, a selective inhibitor of MAO-B. Cells were exposed initially to R (-) and S (+) enantiomers of deprenyl (Deprenyl hydrochloride; Research Biochemicals International Inc. (RBI), Natick, MA). The ability to induce increases in survival of oligodendroglial cells was seen restricted to R (-) -deprenyl and henceforth this enantiomer was used. Experiments involving dose responses of (-)-deprenyl showed that a concentration of 10^{-9} M was most effective in increasing oligodendroglial cell survival. Hence this concentration of deprenyl was used in all subsequent experiments. For deprenyl treatment, cells were washed twice in BM to remove any serum borne survival factors and the media was replaced with BM supplemented with 10^{-9} M (-) -deprenyl (BM-D9). A second control group consisted of cells that were washed and placed in IM supplemented with 10^{-9} M (-) -deprenyl (IM-D9).

2.4 Assessment of Cell Survival by Nuclear Integrity Assay

Cell survival in the cultures was assessed according to the method of Soto and Sonnenschein (1985). After washing and treatment of cells for 24h as described above, cells were harvested, centrifuged at 500 rpm for 5 min and the supernatant removed. Pelleted cells were then lysed with 200µl of 10% Zap-oglobin (Coulter Electronics, FL, USA). The lysing agent is a detergent containing solution that lyses the plasma membrane but leaves the nuclei intact. The lysed solution was collected and vortexed. A small volume (10µl) of the solution was removed and intact nuclei were counted using a haemocytometer. To be counted, nuclei had to show a completely intact smooth outer border. Counts of intact nuclei were pooled for three or four experiments (a total of 15-20 wells were used for each treatment condition).

2.5 Morphological Identification by Methylene Blue Staining and Cell Counts

Methylene blue (3, 7-bis (dimethylamino)-phenothiazin-5-ium chloride), a ribonucleic acid (RNA) binding agent commonly used as an alternative nucleic acid stain, can also be used as a biological staining agent to stain cells in order to observe their morphological features. At 24h, cells were fixed in 2% paraformaldehyde for 15 min at room temperature. Briefly, cells were washed twice with PBS and 100µl of 1% methylene blue (Mallinckrodt Chemical Co., MO, USA) solution containing 1% $AgNO_3$ was added for 10 min to the wells containing coverslips. Subsequently, the coverslips were washed with two changes of distilled water and absolute ethanol, cleared with xylene and mounted on slides with permount.

Cell counts were performed for 25 randomly chosen low (100) power fields on each coverslip. Two randomly generated coordinates on an x-y grid drawn from a uniform distribution of random numbers specified the center of each low power field used for counting. The random numbers were generated on a computer by using Microcal Origin 4.0 program. Initially, to assess the accuracy of the random count method, counts of all the cells on 5% of the coverslips were obtained and compared with randomly generated counts on the same coverslips (see Wadia *et al.*, 1998). Comparison of the counts found by the random method to counts of all the cells on a given coverslip revealed differences of less than ±3% within each slide. In subsequent experiments, different cell types (for example, O-2A progenitors, PROLs, OLs and astrocytes) were morphologically identified on each field and were counted separately using the random counting method for experimental density series- I and II. Interference contrast images of the four oligodendroglial lineage cell types were obtained using a Leitz orthoplan microscope and Metamorph software program (Universal Imaging, PA, USA).

2.6 Nuclear Chromatin Staining with Hoechst 33258

Apoptosis and necrosis can be distinguished on the basis of changes in nuclear chromatin. Light microscopic studies have shown that the characteristic ultrastructural event during apoptotic cell death is the formation of phase dark, crescent shaped and marginated masses of nuclear chromatin (Wyllie, 1987). Necrotic cells on the other hand, show irregular and ill-defined chromatin. Chromatin in necrotic cell death eventually scatters into many loosely associated particles, a phenomenon also known as karyorrhexis (Wyllie *et al.*, 1981).

In order to observe changes in nuclear chromatin during trophic withdrawal and anti-apoptotic compounds, oligodendrocytes at 16 DIV, were exposed for 24 hours to the treatment conditions as described in section 2.4. At 24h, cells were fixed with 2% paraformaldehyde for 15 min at room temperature and subsequently washed three times in PBS. The coverslips were then incubated with 1 μg / ml 2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2-5'-bi-1*H*-benzimidazole trihydrochloride pentahydrate (Hoechst 33258, Molecular Probes) in distilled water for 30 min to visualize nuclear chromatin.

Hoechst 33258 is a supravital bisbenzimide dye, which binds to contiguous A-T bases in DNA. The coverslips were washed three times for 5 min with PBS, mounted in glycerol and viewed with epifluorescence microscopy using 340-380 nm excitation and emission filtration of 430 nm long pass.

2.7 DNA extraction and electrophoresis

DNA was extracted from trophically withdrawn as well as trophically supported oligodendroglial cells and electrophoresed in order to determine the nature of DNA degradation. Oligodendrocytes were cultured in poly-L-lysine coated 100-mm^2 culture dishes at a density of 1×10^6 cells per dish. At 16 DIV, the cultures were exposed to BM, BM-D9, IM and IM-D9 for 24h. The DNA was extracted following the method of Batistatou and Greene (Batistatou and Greene, 1993).

Cells were lysed with 1 ml of lysis buffer (10mM Tris, pH 8.0, 1mM EDTA, 150 mM NaCl and 0.1% SDS). The lysate was then incubated overnight at 65^0C in 20 μg/ml proteinase K (Fisher Scientific, Montreal, Canada) to eliminate contaminating proteins. This was followed by 2-hour (37^0C) incubation in 5 μg/ml DNAse-free RNAse A

(Boehringer Mannheim) to digest RNA. The samples were then extracted twice with a solution of phenol, chloroform, isoamyl alcohol (25:24:1; GibcoBRL). DNA in the lysates was precipitated with 3M sodium acetate (pH 5.2) and ethanol. Subsequently the samples were centrifuged for 30 minutes at 13,000 rpm and a DNA pellet was obtained. The DNA pellet was washed in 70% ethanol and air-dried. The DNA pellet was resuspended in 20µl of TE buffer (10mM Tris, 1 mM EDTA; pH 8.0) and mixed completely.

Each DNA sample (10µl) was mixed with 2 µl of loading buffer (40% Sucrose, 0.25% Bromophenol blue, 0.25% Xylenecyanol) and electrophoresed in a 1.2% agarose (in 1X TAE buffer) minigel at 55V for 1 to 1 1/2 hours. A stock solution of 1 mg / ml ethidium bromide made up to a final concentration of 0.5 µg / ml in TAE (Tris base, acetic acid, EDTA 0.5M; pH 8.6) was used to stain the gels. The gels were photographed on a transilluminator with ultraviolet light using a Polaroid DS-34 camera. Images of the films obtained from the electrophoresis gels were digitized using a Matrox frame grabber and Metamorph software (Universal Imaging, W. Chester, PA).

2.8 *In situ* DNA End Labeling of Oligodendrocytic Cells

The ApopTag™ method utilizes an ApopTag™ *in situ* apoptosis detection kit (Oncor, MD, USA), that detects nuclei with cut 3'- OH ends of DNA. Oligodendrocytes at 16 DIV were treated with BM, BM-D9, IM and IM-D9 for 6, 12, 15, 18 and 24 h. Cells were fixed in 2% paraformaldehyde for 15 min, followed by three washes in 0.1M PBS and permeabilization with 0.05% Tween-20 in PBS for 10 min at room temperature. Endogenous peroxidase was quenched with 1% H_2O_2 in PBS for 5 min at room

temperature. Coverslips were then covered with 1X equilibration buffer for 30 min at room temperature and then incubated with terminal deoxynucleotidyl transferase (TdT) / reaction buffer (76 µl of reaction buffer + 32 µl of TdT enzyme) for 1h at 37°C. A stop wash buffer incubation for 30 min at 37°C was used to terminate the 3'-OH DNA extension reaction. After a PBS wash, the coverslips were incubated with antidigoxigenin peroxidase antibody. After three washes with 0.1M PBS, chromogenic detection was carried out using DAB (1.5 mg / ml) mixed with hydrogen peroxide (H_2O_2; 0.02% v/v) yielding a brown reaction product. Coverslips were dehydrated in ascending grade alcohol series, cleared with xylene and mounted on slides using a drop of permount. ApopTag™ positive nuclei were randomly counted in triplicate (described in section 2.5), for each of the time points, for experimental series- I and II.

2.9 Determination As To Whether (-) -Deprenyl Or Its Metabolites Are Responsible For Oligodendrocyte Anti-Apoptosis

The capacity of the three major metabolites of (-) -deprenyl, namely, (-) -desmethyldeprenyl, (-) -amphetamine and (-) -methamphetamine (Heinonen *et al.*, 1994) to reduce the death of the oligodendroglial cells was assayed using the nuclear integrity assay. Three different general cytochrome P450 blockers - proadifen (Sigma), piperonyl butoxide; Aldrich Chem Co., WI) and 2-methyl-1,2-di(3-pyridyl)-1-propanone (metapyrone; Aldrich) were used to block the cytochrome P450 dependent metabolism of (-) -deprenyl to (-) -desmethyldeprenyl as a means of determining whether a metabolite, rather than (-) -deprenyl was responsible for any changes in survival.

OLs at 16 DIV were exposed to the different drug treatment conditions for 24 h,

following which, the nuclear integrity assay was performed. On the day of the treatment, the cells growing in Nunc 24 well plates were washed twice with base media. Each treatment well received 50μl of the appropriate drug solution and the total volume in the well was made up to 500μl with either BM or IM.

Nuclear counts were obtained from quadruplicate wells per treatment condition and the experiment was repeated twice. OLs were exposed to the following treatment conditions: 1) IM, 2) BM, 3) BM-D9, 4) (-) -Desmethyldeprenyl (BM-Ds9), 5) IM-D9 and 6) IM + 10^{-9} M (-)-Desmethyldeprenyl (IM-Ds9).

The treatment conditions with the three general cytochrome P450 blockers were: 1) BM + Proadifen (2.5, 10, 25 μM), 2) BM-D9 + Proadifen (2.5, 10, 25 μM), 3) BM-Ds9 + Proadifen (2.5, 10, 25 μM), 4) BM + Metapyrone (50 μM), 5) BM-D9 + Metapyrone (50 μM), 6) BM-Ds9 + Metapyrone (50 μM), 7) BM + Piperonyl butoxide (100 μM), 5) BM-D9 + Piperonyl butoxide (100 μM), 6) BM-Ds9 + Piperonyl butoxide (100 μM).

The effects of the other two metabolites of (-) -deprenyl, namely, (-) -methamphetamine and (-) -amphetamine on oligodendroglial cell survival were also tested. (-) -Methamphetamine (Methamphetamine hydrochloride; RBI) was used at three doses (10^{-5} M, 10^{-7} M, 10^{-9} M) in combination with 10^{-9} M (-) -deprenyl while a single dose of 10^{-5} M (-) -methamphetamine was used with 10^{-9} M (-) -desmethyldeprenyl.

(-) -Amphetamine (Amphetamine hydrochloride, RBI) was used in four concentrations (10^{-3} M, 10^{-5} M, 10^{-7} M, 10^{-9} M) with (-) -deprenyl (10^{-9} M) while a single concentration of 10^{-5} M (-) -amphetamine was used in combination with (-) -desmethyldeprenyl (10^{-9} M).

2.10 Determination of the Relationship between New Protein Synthesis and Apoptosis in Oligodendrocytes

I performed experiments to determine whether oligodendroglial apoptosis after insulin, IGF-I and serum withdrawal and the effects of (-) -desmethyldeprenyl were related to a transcriptional- or a translational-dependent mechanism.

OLs at 16 DIV were washed twice with base media and exposed either to actinomycin, a transcriptional blocker or cycloheximide, a translational blocker for 24 h. Actinomycin was used at 1, 2.5 and 50 μg / ml and cycloheximide at 1, 5, 10 and 50 μg / ml. Actinomycin and cycloheximide were either used with base media alone or in combination with 10^{-9} M (-) -desmethyldeprenyl. Each treatment well received 50μl of the drug solution and the total volume of the well was made up to 500μl with base media.

Counts of intact nuclei, obtained from quadruplicate wells per treatment condition were used to quantitate the effects of the transcriptional and translational blockers. Each experiment was repeated twice for experimental density series-II.

2.11 Analysis of Changes in OL Protein Levels

2.11.1 Protein Extraction

O-2A progenitor cells were plated at a density of 1 x 10^6 / 100 mm culture dishes. The progenitor cells were allowed to differentiate into PROLs and OLs in the presence of insulin and IGF-I. At 16 DIV, cultures were exposed to BM, IM , BM-D9 or BM-Ds9 for 24 h. Subsequently, cells were washed twice with 0.1M PBS, pH 7.4 and harvested with

a Falcon cell scraper. After centrifugation at 2000 x *g* for 5 min in a Mistral 2000 centrifuge, the cells were incubated in 100µl of cell lysis buffer (25 mM Tris, pH 8.0, 0.5% Triton-X-100, 1mM EDTA, 10mM PMSF, 5mM Benzamidine, 5µM Leupeptin). The lysate was homogenized with a plastic homogenizer. The lysate was centrifuged at 2000 x *g* for 5 minutes at 4°C. The supernatant containing the protein fraction was collected. A small volume of the protein solution was used to estimate the protein concentration. The rest of the protein solution was stored at -20°C until use.

2.11.2 Estimation of Protein Concentration

Total protein of the aliquot was determined by spectrophotometry based on the Bradford dye binding procedure (Bradford, 1976) using Coomassie Brilliant Blue (Pierce, Rockford, IL). Coomassie Brilliant Blue dye binds primarily to basic or aromatic residues on proteins and in the presence of the protein converts from cationic (red) to the anionic (blue) form by successive protonations. This conversion was quantitated by a Hitachi spectrophotometer at a wavelength of 595 nm. The assay was carried out by measuring the binding of the dye to the unknown protein. The protein - dye binding was then compared to different amounts of a standard protein, bovine serum albumin (BSA). Proteins with a molecular weight greater than 3 - 5 kDa are sensitively quantitated by the Bradford protein assay.

2.11.3 SDS - PAGE Electrophoresis

The protein samples obtained from the cultures after a 24-hour treatment period were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-

PAGE) in duplicate. The SDS-PAGE electrophoresis separates proteins based on molecular size as they move through the polyacrylamide gel matrix from the cathode towards anode. The percentage of acrylamide in the separating gel depends on the molecular size of the protein electrophoresed. Proteins smaller than 50 kDa like MBP and PLP were resolved on a 10% SDS-PAGE discontinuous gel. A 30% stock acrylamide solution was made up. A separating and stacking gel mix was prepared using stock acrylamide, water, separating or stacking gel buffer, N,N,N',N'-tetramethyl ethylenediamine (TEMED) and ammonium persulfate (APS). APS was always made fresh for each experiment. The glass plate sandwich with spacers was assembled and placed in a Bio Rad Mini Protean II gel apparatus. Using Pasteur pipets, the separating gel was poured and the top of the gel was covered with a layer of isobutyl alcohol. The separating gel was allowed to polymerize for 1h at room temperature. The overlaid isobutyl alcohol on the separating gel was poured out and the stacking gel was poured. Subsequently, a 10 well Teflon comb was placed in the stacking gel and the gel was allowed to polymerize. The protein samples were solubilized in sample buffer (glycerol, Tris, pH 6.8, 0.01% Bromophenol blue, 20 mM Dithiothreitol, SDS) in a 1:1 ratio, sample to buffer. The protein samples in tubes were transferred to a 100°C water bath for 5 minutes to inactivate proteases. The samples were then loaded carefully as a thin layer on the bottom of the polymerized wells. An electrophoresis buffer (Glycine, Tris, pH 8.3, SDS) was used to fill up the upper and lower buffer chambers. A Bio-Rad model 250 / 2.5 power supply was used to resolve the samples at 70 volts through the stacking gel and at 100 volts through the separating phase of the gel.

2.11.4 Detection of Proteins By Western Blotting Immunodetection method

The oligodendroglial proteins separated by SDS-PAGE were transferred to Hybond-C nitrocellulose membrane (Amersham Life Science) which is optimized for protein transfer, using a Bio-Rad Mini Protean II transfer apparatus. All the filter papers and nitrocellulose membranes were first equilibrated in a transfer buffer (Glycine, Tris, SDS, 20% Methanol). The transfer apparatus was connected to a power supply and the protein transfer was carried out at 100 mA for 3 h. After the transfer period, the membrane was removed and proteins were visualized by staining for 5 min with Ponceau S stain (0.5% Ponceau S and 1% acetic acid). Ponceau S staining is primarily used to verify transfer efficiency. The membrane was destained with distilled water.

Prior to immunodetection, non-specific binding sites on the membrane were blocked by immersing it in a blocking buffer (5% Carnation nonfat dry milk in 0.1M PBS) for an hour at room temperature with shaking. The membrane was rinsed with 0.1M PBS twice and incubated in a primary antibody overnight at 4^0C. The primary antibodies used were diluted in 1% nonfat dry milk in PBS. The following monoclonal primary antibodies were used: rat anti-MBP (82-87 amino acid region) (1:400; Serotec Ltd., UK)and rat anti-PLP (1:200; Immuno Diagnostics, Inc., MA). Following overnight incubation in primary antibodies, the membranes were washed four times with a wash buffer consisting of Tris Buffered Saline (TBS) containing 0.1% Tween-20. The blot was incubated in rat, rabbit or mouse IgG-horseradish peroxidase-conjugated secondary antibody for an hour at room temperature. The secondary antibody was used at a dilution of 1:500 in 1% nonfat dry milk. The blots were washed five times with TBS containing

0.1% Tween-20.

To visualize the antigen-antibody interaction in the immunoblots, an enhanced chemiluminescent method (ECL) was used. The horseradish peroxidase labeled ECL reaction involves a substrate solution containing luminol. Oxidized luminol substrate gives off a blue light that can be trapped on a film. For the ECL reaction, a Supersignal™ Substrate (Pierce, Rockford, IL) working solution containing luminol / enhancer solution and a stable peroxide solution in a 1:1 v / v ratio was used. The blots were placed in the Supersignal™ Substrate working solution for 5 minutes at room temperature with shaking. A sufficient amount of the solution was used to ensure that the blot remained completely wetted. After five minutes, the blot was removed from the Supersignal™ Substrate working solution and placed in a plastic wrap. Any excess of the working solution or bubbles between the blot and the plastic wrap was removed. The blots were placed against a Kodak Biomax-MR double emulsion film in an autoradiographic cassette. Typically, blots were exposed to the films for 1-10 seconds and the films were developed.

2.12 Assessment of Mitochondrial Membrane Potential in Oligodendroglial Cells

2.12.1 CMTMR Estimation of Mitochondrial Membrane Potential

The $\Delta\Psi_M$ was assessed by the addition of a lipophilic, potentiometric dye, chloromethyl-tetramethylrhodamine methyl ester (CMTMR, Mitotracker Orange™ - Molecular Probes, Eugene, OR). The CMTMR dye reacts with the thiol groups on proteins and peptides to form aldehyde fixable conjugates. Entry to the negatively charged mitochondrial matrix compartment is proportional to the difference in the

membrane potential. Therefore, the CMTMR fluorescence labels the mitochondrial membrane potential generated due to the negativity difference between the mitochondrial matrix and the outside of the mitochondria. Upon cell fixation, CMTMR remains in the mitochondria and is not washed out unlike other mitochondrial potentiometric dyes like rhodamine123.

Oligodendrocytes at 16 DIV were placed in trophically deprived BM for 18h or treated with BM-D9 or BM-Ds9. Other treatments of cultures included exposure to the general cytochrome P450 blockers as well as the metabolites of (-)-deprenyl as previously described in section 2.8 of this thesis. At 18 h after trophic withdrawal or drug treatments, the media in each well was replaced with similar media containing 138 nM CMTMR. The cells were incubated in CMTMR at 37°C for 15 min. The cells were then fixed with 4% cold paraformaldehyde on ice for 30 min and washed twice with 0.1M PBS. Consequently, the coverslips were mounted on glass slides with Aquamount (Gurr, England).

2.12.2 Confocal Microscopy

Individual mitochondria labeled with CMTMR were resolved by confocal microscopy. A Leica true confocal scanning (TCS) 4D microscope coupled to an argon-krypton laser (Omnichrome, USA) was used for cell imaging. A pinhole size of 20 with an excitation filter wavelength of 568 nm and an emission filter wavelength of 590 nm was used to image the CMTMR labeled mitochondria. Each field of the coverslip was imaged using an oil-immersion, 100X, 1.3 N.A. objective at 512 by 512 by 8 bits per pixel resolution, background offset of -1 and line averaged 32 times in bi-directional line

scan mode. Care was taken not to exceed the dynamic range of the gray value display. The confocal images obtained were saved on an optical drive in a tagged image file format (TIFF).

2.12.3 Measurement of CMTMR Fluorescence Intensity

Fluorescence intensity of individual mitochondria was measured using the Metamorph™ program. Metamorph™ compiles measurements of objects in a 8-bit image ranging from gray values 0 (background) and 255. In order to obtain fluorescence intensity measures, a rectangular region tool of 2x2 pixels was used to measure two randomly selected areas within each mitochondrion. A total of ten mitochondria obtained equally from all the regions of each cell were measured within each image. About 20 cells were analyzed for each treatment condition thereby giving a sum of about 400 mitochondrial fluorescence intensity measures. The raw values were then further graphed and analyzed with the Microcal Origin 4.1 program. Frequency count was performed on the data, which classified the data in ordered bins. A frequency distribution was obtained with lower bin value set at 0 and higher bin value set at 260. The data are presented as frequency distribution histograms. The mean ± SEM of the intensity of the mitochondrial fluorescence is also presented along with the histograms.

2.13 Statistical Analysis

In order to statistically evaluate the data, the individual measurements of data from different treatment groups were first analyzed using Statistica™ software (StatSoft) to carry out two-tailed independent sample t-testing, a commonly used method to evaluate

the differences in means between two groups. The t-testing method uses the sample variance as an estimate of the population variance and relies on the assumption that the data is derived from a population that is normally distributed and that there is homogeneity of variances between groups being compared. The shape of the normal distribution is the characteristic "bell curve" that is defined by a function that has mean and standard deviation as its parameters.

Analysis with parametric methods such as the t-test may not provide valid results, especially if the variables analyzed within the population do not conform to a normal distribution. Secondly, the t-test is not reliable where the variances of the independent samples within the population are not homogeneous. Hence the data was statistically analyzed by a non-parametric test that does not rely on parameter estimation and distribution assumption. Another characteristic of non-parametric tests is that they are more sensitive to the medians rather than the means. Since the tests rank order the data, they offer a test of differences of differences in central tendency that are not affected by one or a few very extreme scores or outliers. The data presented in this thesis was therefore rank ordered and compared in a pairwise fashion using Statistica™ software to perform non-parametric Mann Whitney U testing (Siegel, 1956). The Mann-Whitney U test is computed based on rank sums rather than means.

The data presented in this thesis was analyzed both by t-test for independent samples and the Mann-Whitney U test and presented as 'p-values'. There are conventions which are informally based on general research experience that assign levels of significance, namely $p \leq .05$ (a 5% probability of error) or $p \leq .01$ (a 1% probability of error). $p \leq .05$ or the 5% probability of error indicates that 95 out of a 100 times the same

result is obtained while 5 times a different result could occur. $p \leq .01$ or the 1% probability of error indicates that 99 out of a 100 times the same result is obtained while once a different result could occur. In order to avoid arbitrariness in the final decision of upto which level of significance the results will be rejected as invalid, the data is presented in this thesis as the raw p-values themselves which provide consistent supportive evidence of the reliability of the data.

3.0 Results

3.1 Differentiation and Maturation of O-2A Progenitors

Phase contrast microscopy revealed cells with a round cell body of about 10 μm in diameter with a round nucleus and two long thin bipolar processes extending from the cell body at about an angle of 180 degrees to each other. Those cells were found to be immunopositive for an A2B5 antibody which is characteristic of O-2A progenitor cells (Eisenbarth *et al.*, 1979). Figure 1A shows a phase contrast image of a typical O-2A progenitor, which is marked by the white arrow. The interference contrast image in figure 2A shows four cells with the bipolar morphology that are immunopositive for A2B5. Only cells with the bipolar morphology were found to be A2B5 immunopositive. On 12 DIV, 24 hours after the cultured cells were removed from the astrocyte feeder layer and replated on coverslips at 11 DIV, A2B5 immunopositive cells accounted for more than 75% of the cultured cells.

By 13 DIV, phase contrast microscopy revealed increasing numbers of cells with three to four processes emanating from the cell body. Figures 1B and 1C present phase contrast images of the cells. The white arrows in the figures mark typical examples. These cell bodies and processes of those cells were found to be immunoreactive for the antibodies CNPase-I and GC which have been shown to identify PROLs (Knapp, 1988; Ranscht, 1982). Cells with the multiple processes did not immunoreact for the A2B5 antibody or for antibodies to MBP. By 14 and 15 DIV, the PROLs constituted a major proportion of the cells in the cultures.

Fig. 1. Progressive maturation of oligodendrocytes *in vitro.* Phase-contrast microphotographs of oligodendrocytes after 12 (A), 13 (*B*), 14 (C) and 16 days (D) of culture. O-2A progenitor cells isolated from postnatal day 2 rat cortex were grown in a chemically defined media supplemented with insulin (5 μg/ml) and IGF-I (2.5 ng/ml). Bipolar O-2As (A) differentiate into multipolar PROLs (B, C) which develop into OLs (D) bearing an extensive network of branching processes. The cell marked by the white arrow in A is a typical O-2A while typical multiprocessed PROLs are shown in (B) and (C) marked by white arrows. The white arrow in panel (D) marks a typical example of an OL. A 16 DIV culture constituted about 2% O-2As, 32% PROLs and 62% OLs and 4%AST.

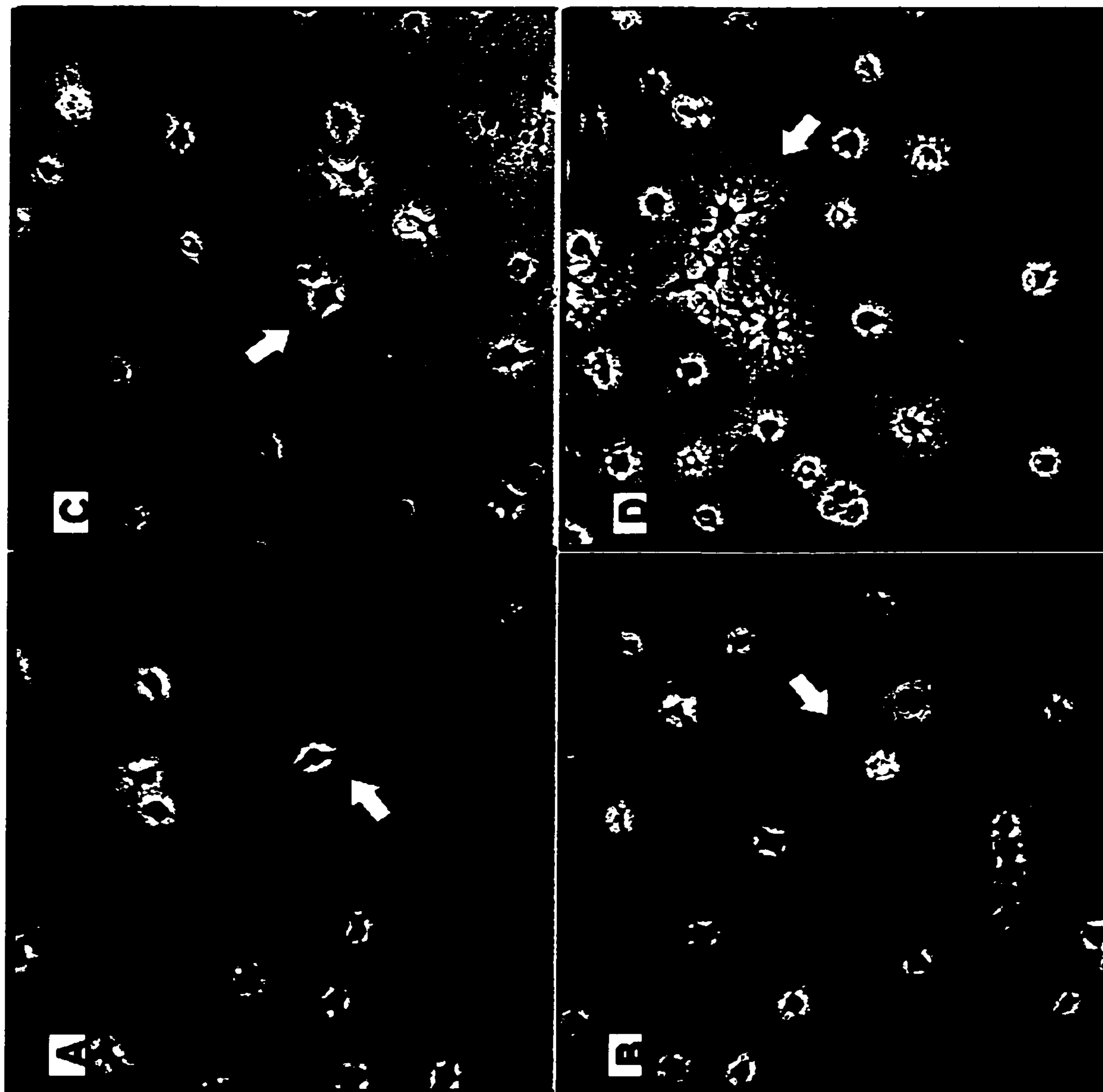


Figure 1

By 15 and 16 DIV, the PROLs elaborated more complex processes. These cells were highly branched and had 6 to 8 main processes. As shown by the typical cell marked by the white arrow in figure 1D, the interlacing small secondary and tertiary processes gave the processes a web-like appearance. Cells with the web-like processes were immunopositive for MBP (shown in figure 2D) and PLP (shown in figure 2E). MBP (Lees, 1984) and PLP (Bartlett *et al.*, 1988) have been shown to be markers for OLs. Staining with the MBP antibody also revealed the presence of the immunoreaction product in the nucleus as well as the perikarya of the differentiated OLs. This observation of the nuclear localization of MBP as a specific phenomenon gains support from another study, which has demonstrated heterogeneity in the localization of MBP in OLs (Hardy et al., 1996). The study of Hardy et al. (1996) has shown that all the four isoforms of MBP, the 21.5, 18.5, 17 and 14 kD are present in the nucleus of the OLs. It is thought that the nuclear MBP is involved in the regulation of mRNA processing and gene expression prior to the onset of the myelination process. Some of the MBP positive cells were also found to have flat MBP positive membranes attached to the web-like processes.An intense staining of PLP was found in the cell body.

A small proportion of cells displayed a flattened morphology with a large round nucleus. These cells immunoreacted to the antibody glial fibrillary acidic protein (GFAP) as shown in figure 2F. The cells also possessed GFAP positive reticulated somal areas. These observations led to the identification of the GFAP positive cells as ASTs (Raff *et al.*, 1979). Less than 1% of the cells in culture showed a non-process bearing amoeboid morphology and were identified as microglia. The controls run in parallel for each

antibody revealed no staining suggesting that the immunostaining was specific for each antibody used.

This is the first study to demonstrate a *in vitro* method which generated over 60% differentiated OLs. This method used to isolate and differentiate OL cells in primary culture differs from those previously reported (Barres *et al.*, 1992) which generated undifferentiated or partly differentiated oligodendroglial cells as opposed to the high percentage of fully differentiated OLs obtained in this study.

Fig. 2. Immunocytochemical characterization of the cells of the oligodendroglial lineage. O-2A progenitors on 12 days in vitro (DIV) were labeled with an antibody to a surface tetraganglioside A2B5 (A). PROLs express CNP (B) and GC (C) on 13 DIV. OLs on 16 DIV reacted with anti-MBP (D) and anti-PLP protein (E) antibodies. Note MBP expression in the cell body and processes. An intense staining of PLP was found in the cell body. ASTs were identified by staining with an antibody to GFAP (F). GFAP positive ASTs show reticulated somal areas.

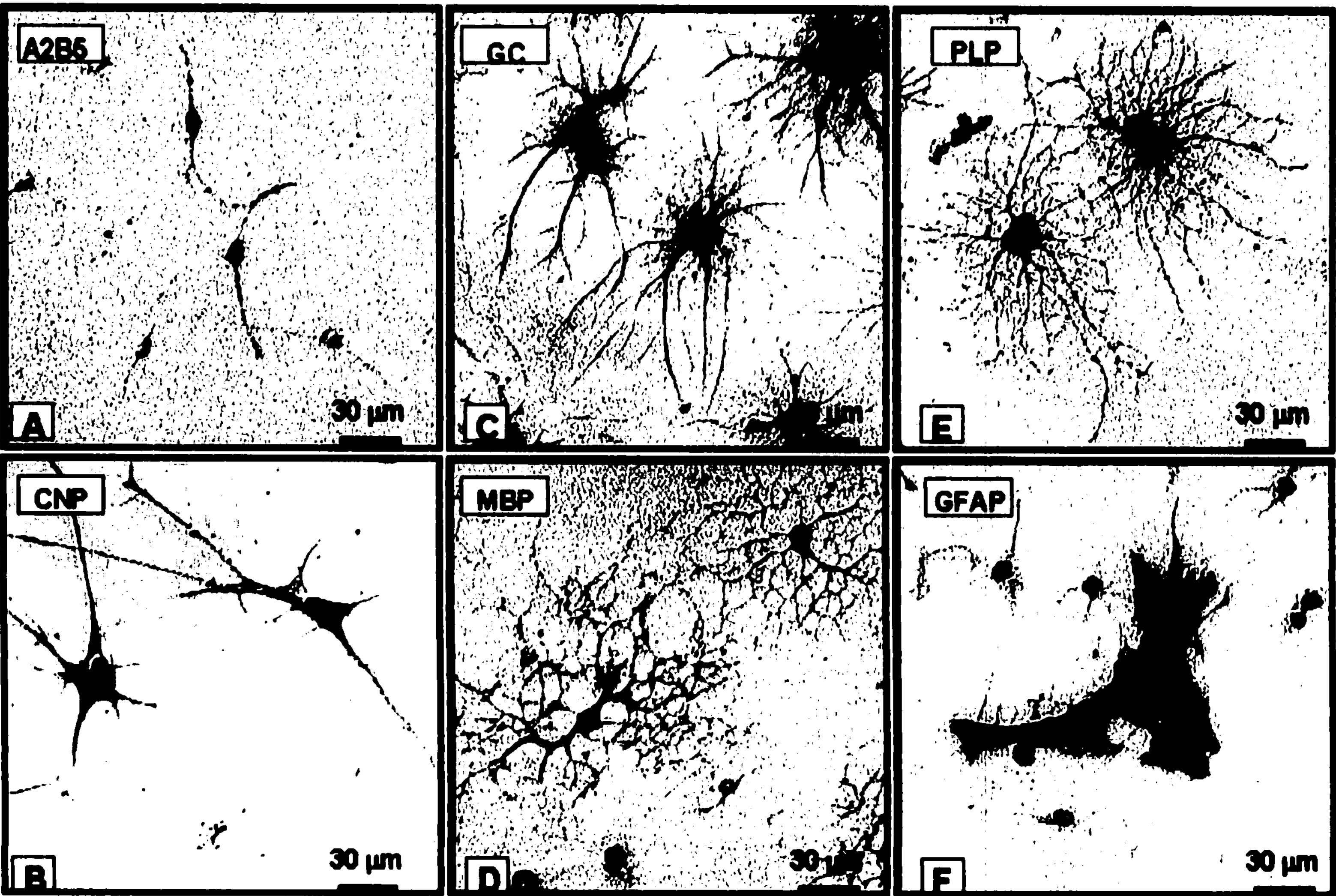


Figure 2

Typically, 16 DIV culture comprised of 4% AST, 2% O-2A, 32% PROL and 62% OL (see figure 5 below). Since the differentiated PROL and OLs accounted for more than 93% of the total cells at 16 DIV, all the experiments reported in this thesis used 16 DIV cultures. The progressive increase in OLs and PROLs and the progressive decrease in O-2As between DIV 12 and 16 and the small numbers of ASTs at 16 DIV showed that the base media, with IGF-I and insulin added, favored the differentiation of O-2As along the oligodendrocyte pathway rather than along the type 2 astrocyte pathway. The methods reliably provided large number of identifiable PROLs and OLs at 16 DIV. The studies of apoptosis were therefore carried out at 16 DIV.

3.2 Decreased Survival of Oligodendroglial Cells after Insulin and IGF-I and Serum Withdrawal

Cultures on 16 DIV were washed free of serum, insulin and IGF-I. The wash media was then replaced with BM. Control cultures were placed in IM. The washing and replacement with defined media served to suddenly withdraw IGF-I and insulin from the cells. The cells were exposed to either BM or IM for a 24 h period after which cell survival was estimated using counts of intact nuclei. Two independent series of experiments were carried out at different plating densities.

3.2.1 Counts of Intact Nuclei

Insulin, IGF-I and serum withdrawal resulted in a decrease in numbers of intact nuclei at 24h after washing. In experimental series-I (plating density of $5.3x10^3$ cell/mm^2), counts of intact nuclei decreased to 54.8% (bar labeled BM in figure 3A1) compared to the intact nuclear counts for cells that were washed and replaced in media with IGF-I and insulin (bar labeled IM in figure 3A1). A similar decrease to 51.4% was observed in experimental series-II (plating density of $15.8x10^3$ cell/mm^2) as shown by the bars labeled IM and BM in figures 3A2. Table 1 presents the statistical probabilities for experimental series I and II. A chi-square evaluation of the data presented in this thesis showed that the data did not fit to a normal distribution and Levene's testing for homogeneity of variances showed that most pairs of samples were not homogeneous. Hence the data was compared using a non parametric Mann-Whitney U test. Each experimental series for the total intact cell counts consisted of three or four independent experiments.

Experiments were carried out to determine whether (-) -deprenyl altered the reduction in the counts of intact nuclei induced by insulin, IGF-I and serum withdrawal. After washing and placement in insulin and IGF-I free media supplemented with 10^{-9} M (-) -deprenyl (bars labeled BM-D9 in figure 3A1), the numbers of intact nuclei were 119% of those in IM in experimental series- I so that (-) -deprenyl increased the number of cells after insulin, IGF-I and serum withdrawal by 217% (compare bars labeled BM-D9 and BM in figure 3A1). The BM decrease to 84% of IM was smaller in experimental series- II but BM-D9 still showed an increase of 164% compared to BM (compare BM

and BM-D9 bars in figure 3A2). When cells were placed in IM with 10^{-9} M (-) -deprenyl (IM-D9 bars), the number of intact nuclei were 136% compared to IM in experimental series- I) and 102% compared to IM for experimental series- II (figure 3A2).

According to the counts of intact nuclei, treatment with 10^{-9} M (-) -deprenyl induced a significant increase in numbers of intact nuclei both for cells washed and replaced in media with IM and those washed and placed in BM (see table 1).

Fig. 3. Withdrawal of insulin, IGF-I and serum decreased the number of intact nuclei and intact cells. (-) -Deprenyl increased the survival of oligodendrocytes in a serum free, insulin and IGF-I deprived base medium (BM). After 16 DIV, cells were washed and incubated in BM or BM supplemented with 10^{-9} M (-) -deprenyl (BM-D9) for 24h. For controls, washed cells were replaced in media (IM) containing insulin (5 μg/ml) and IGF-I (2.5 ng/ml) or IM supplemented with 10^{-9} M (-) -deprenyl (IM-D9). Intact nuclei were counted using a haemocytometer after Zap-oglobin lysis. Total cell numbers indicate counts of intact methylene blue stained cells by a random method (see Materials and Methods). Data shown are mean ± SEM of nuclei (A1, A2) or cells (B1, B2) relative to IM (data are expressed as % relative to IM) counted in two experimental series in which the cells were grown at seeding densities of 5.3×10^3 cells / cm^2 (experimental series - I) and 15.8×10^3 cells / cm^2 (experimental series - II). The data shown are representative of experiments repeated in quadruplicate (n=20) for the two experimental series. ***$P < 0.001$, *$P < 0.05$ compared with IM, Mann-Whitney U test.

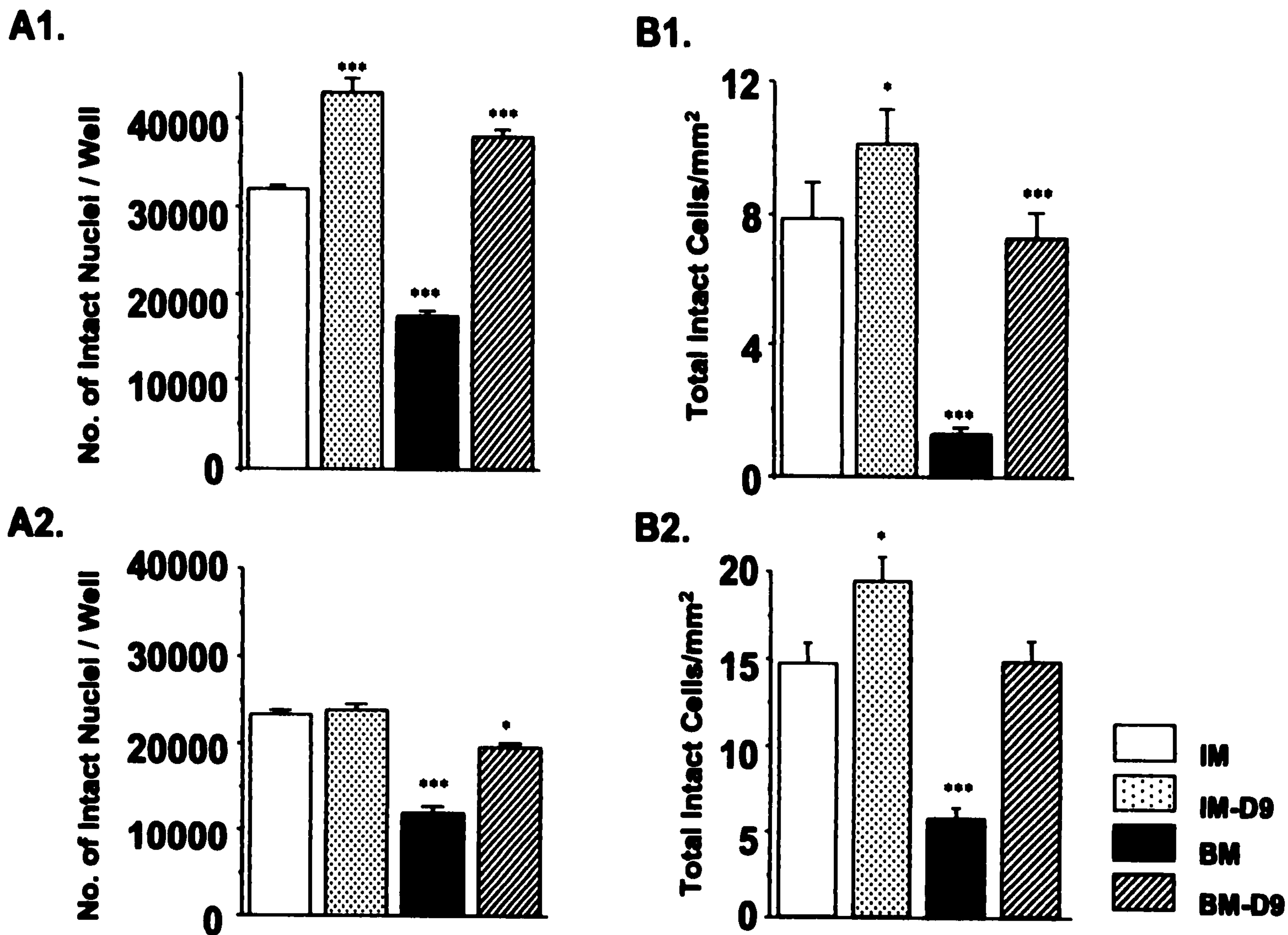


Figure 3

Table 1. Statistical testing of data for intact nuclear and cell counts

Experimental Series - I			Experimental series - II		
	T- test for independent samples			T- test for independent samples	
Treatment	Intact nuclear counts	Intact cell counts	Treatment	Intact nuclear counts	Intact cell counts
IM-D9 / IM	1.9×10^{-5}	1.0×10^{-1}	IM-D9 / IM	5.3×10^{-1}	3.2×10^{-3}
BM / IM	1.1×10^{-13}	4.8×10^{-10}	BM / IM	1.2×10^{-7}	2.1×10^{-17}
BM-D9 / IM	2.8×10^{-4}	6.5×10^{-1}	BM-D9 / IM	9.9×10^{-5}	9.5×10^{-1}
BM-D9 / BM	1.4×10^{-13}	5.9×10^{-14}	BM-D9 / BM	4.7×10^{-7}	2.1×10^{-16}
	Mann - Whitney U test			Mann - Whitney U test	
Treatment	Intact nuclear counts	Intact cell counts	Treatment	Intact nuclear counts	Intact cell counts
IM-D9 / IM	1.1×10^{-5}	1.4×10^{-3}	IM-D9 / IM	3.9×10^{-1}	2.0×10^{-2}
BM / IM	1.3×10^{-8}	1.4×10^{-4}	BM / IM	6.6×10^{-4}	4.9×10^{-11}
BM-D9 / IM	7.1×10^{-5}	9.4×10^{-2}	BM-D9 / IM	1.3×10^{-3}	6.7×10^{-1}
BM-D9 / BM	1.3×10^{-8}	7.8×10^{-10}	BM-D9 / BM	1.6×10^{-4}	3.7×10^{-9}

The increase in numbers of intact nuclei for cells that were washed and replaced in media with IM was compatible with two interpretations: 1) that (-) -deprenyl induced the increased number of intact nuclei in cells that were washed and placed in media BM by increasing the replication rates of the cells, and 2) that (-) -deprenyl decreased the death of the cells caused by insulin, IGF-I and serum withdrawal and also decreased a baseline death process that was present in cells washed and replaced in IM.

3.2.2 Total Counts of Methylene Blue Stained Cells

The counting of methylene blue stained cells plated on coverslips resulted in data, which paralleled that found for counts of intact nuclei above. In experimental series-I, washing and placement of cells in BM (bar labeled BM in figure 3B1) decreased in the intact cell counts of 16.2% with respect to cells that were washed and replaced in media IM (bar labeled IM in figure 3B1). Similarly, the BM intact cell counts decreased to 39.4% of IM in experimental series-II (figure 3B2). Similar to the intact cell counts, treatment with (-) -deprenyl (BM-D9) increased the number of intact methylene blue stained cells to 576% of those in BM in experimental series- I (figure 3B1) and 255 % of those in BM in experimental series- II (figure 3A2).

The number of intact methylene blue stained cells in IM-D9 were 129% (experimental series- I) and 131% (experimental series- II) relative to IM (figures 3B1, and 3B2). The data obtained from the intact nuclear counts and cell counts showed that (-)-deprenyl increased the numbers of oligodendroglial cells when administered in combination with either BM or IM. Like the data for counts of intact nuclei, the changes

induced by (-)-deprenyl could result from an increase in replication or a decrease in cell death.

3.2.3 Intact Cell Counts for Individual Cell Types in Cultures

The characteristic morphologies for the O-2A, PROL, OL and AST cells as revealed by immunocytochemical identification above allowed the identification of the cell types on methylene blue stained coverslips. Figure 4A-D shows typical methylene blue stained O-2A, PROL, OL and AST cell types.

On washing and placement in BM, the counts of all four cell types showed a significant reduction in numbers (see figure 5). In experimental series-I, the numbers for BM, when compared to IM, were 30.3%, 69.2%, 14.0%, 15.1% for ASTs, O-2As, PROLs and OLs respectively (figure 5A). As above for the total cell counts, relatively less cell death was observed in experimental series-II. The survival in BM compared to IM for experimental series-II was 71.2%, 42.2%, 37.3%, 35.0% for ASTs, O-2As, PROLs and OLs respectively (figure 5B). Note that among the four cell populations, maximum decreases in numbers were seen in the more differentiated PROL and OL cells.

(-) -Deprenyl increased the numbers of all the four types of cells in the IGF-I and insulin withdrawn cultures. For BM-D9, cell numbers increased compared to those in BM to 570% for ASTs, 244% for O-2As, 739% for PROLs and a 565% for OLs in experimental series-I (figure 5A) and to 132% for ASTs, 176% for O-2As, 275% for PROLs and 291% for OLs for experimental series II (figure 5B).

Fig. 4. Morphological identification of the oligodendroglial cells identified by methylene blue staining. O-2As exhibit a typical bipolar morphology with two long thin processes extending from the cell body (A) while the PROL show a multipolar appearance with 3-5 processes (B). A complex network of secondary and tertiary processes branching off form the primary process characterizes OLs (C). AST display a flattened morphology with a larger cell body and nucleus compared to O-2As, PROLs and OLs (D).

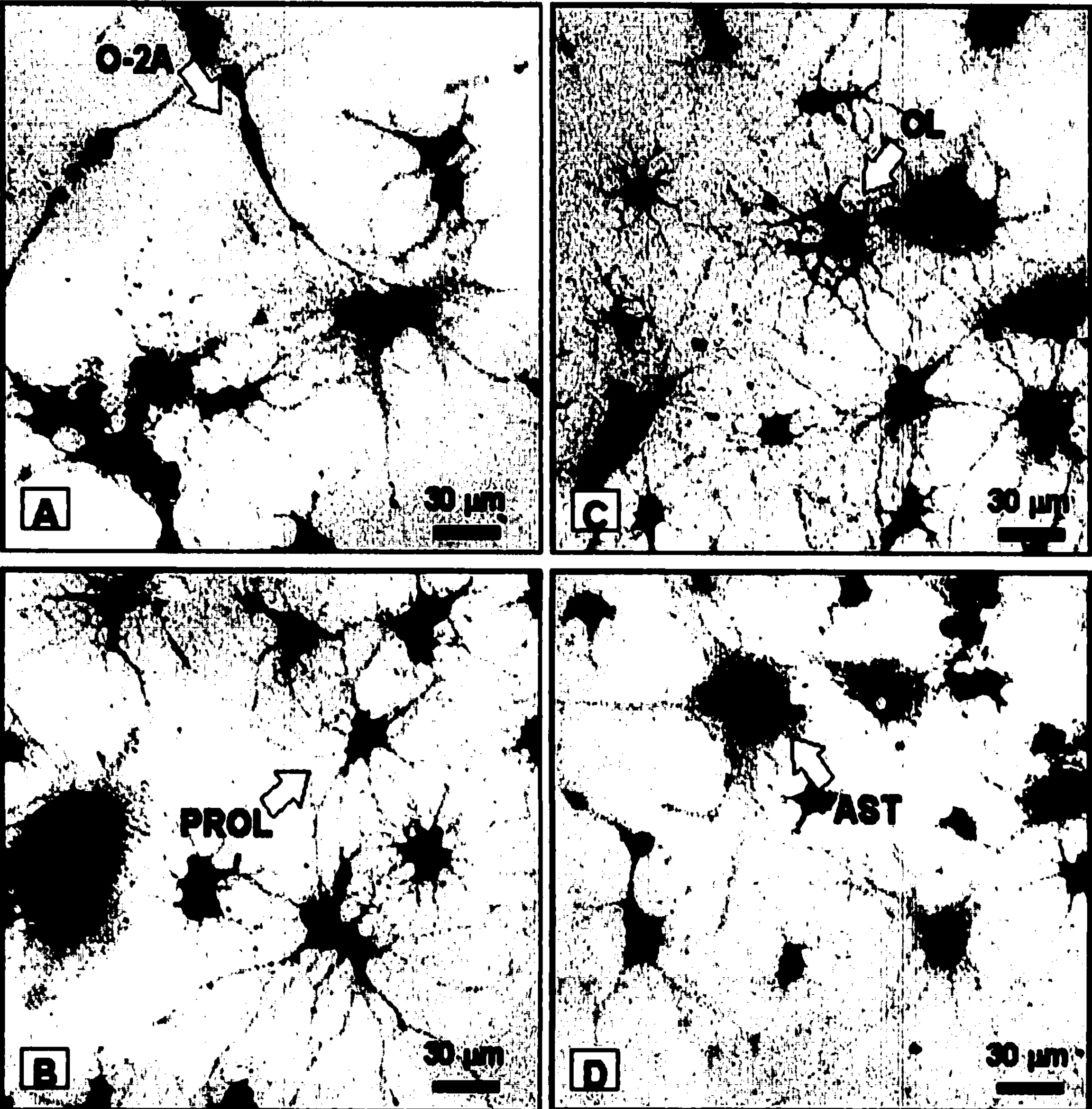


Figure4

Fig. 5. Effects of insulin, IGF-I and serum withdrawal and treatment with R (-) - deprenyl on oligodendroglial cell numbers. Cells grown on coverslips on 16 DIV were incubated in BM, BM-D9, IM or IM-D9 for 24 hours. After the treatment period cells were stained with 1% methylene blue solution containing 1% $AgNO_3$. Methylene blue stained positive cell types were morphologically identified as O-2As, PROLs, OLs or AST and counted in 25 randomly chosen low (100) power fields on triplicate coverslips (n=50). Results are expressed as mean ± SEM for two separate experiments for experimental series - I (A) and experimental series -II (B). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with IM, Mann-Whitney U test.

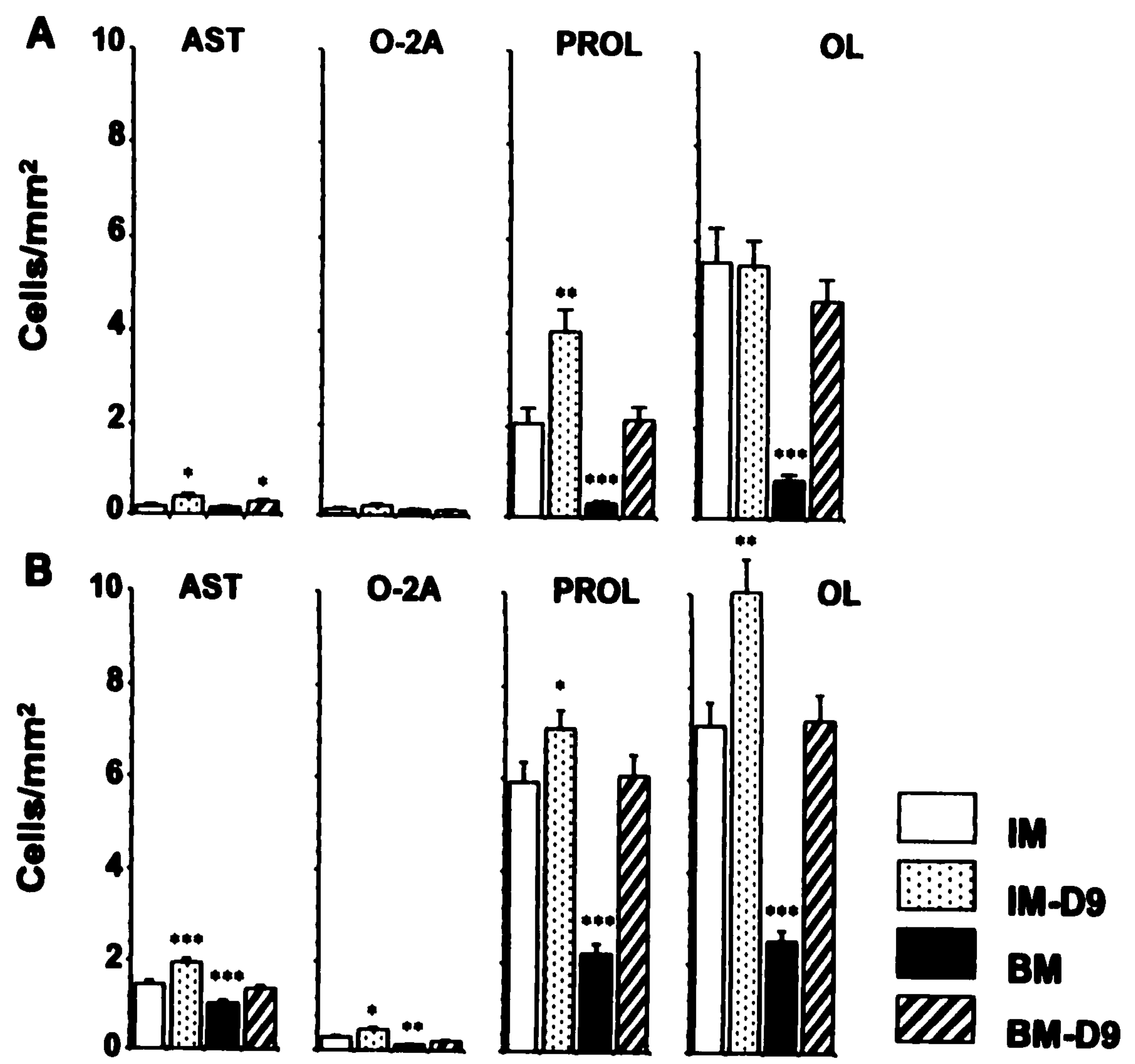


Figure 5

Table 2. Statistical testing of data for counts of individual cell types

Experimental Series - I

T - test for independent samples

Treatment	AST	O-2A	PROL	OL
IM-D9 / IM	4.2×10^{-3}	1.1×10^{-2}	7.2×10^{-4}	9.5×10^{-1}
BM / IM	8.9×10^{-3}	5.0×10^{-1}	1.0×10^{-6}	6.0×10^{-9}
BM-D9 / IM	5.8×10^{-2}	2.3×10^{-1}	8.6×10^{-1}	3.1×10^{-1}
BM-D9 / BM	7.0×10^{-6}	5.4×10^{-2}	2.7×10^{-9}	3.0×10^{-12}

Mann - Whitney U Test

Treatment	AST	O-2A	PROL	OL
IM-D9 / IM	2.7×10^{-2}	8.6×10^{-2}	1.3×10^{-3}	6.5×10^{-1}
BM / IM	1.0×10^{-1}	9.3×10^{-1}	5.7×10^{-4}	4.8×10^{-7}
BM-D9 / IM	4.5×10^{-2}	3.1×10^{-1}	5.3×10^{-1}	6.7×10^{-1}
BM-D9 / BM	1.0×10^{-4}	2.5×10^{-1}	9.7×10^{-8}	9.5×10^{-9}

Experimental Series - II

T - test for independent samples

Treatment	AST	O-2A	PROL	OL
IM-D9 / IM	2.1×10^{-4}	1.0×10^{-2}	5.9×10^{-2}	1.1×10^{-3}
BM / IM	1.5×10^{-4}	2.7×10^{-4}	3.2×10^{-1}	1.3×10^{-12}
BM-D9 / IM	4.5×10^{-1}	1.2×10^{-1}	8.1×10^{-1}	8.6×10^{-1}
BM-D9 / BM	1.6×10^{-3}	1.3×10^{-2}	1.3×10^{-12}	1.1×10^{-12}

Mann - Whitney U Test

Treatment	AST	O-2A	PROL	OL
IM-D9 / IM	5.5×10^{-5}	2.4×10^{-2}	3.3×10^{-2}	3.5×10^{-3}
BM / IM	3.6×10^{-4}	5.9×10^{-3}	4.2×10^{-1}	2.1×10^{-11}
BM-D9 / IM	4.5×10^{-1}	3.1×10^{-1}	8.1×10^{-1}	9.6×10^{-1}
BM-D9 / BM	3.1×10^{-3}	6.5×10^{-2}	4.0×10^{-11}	6.7×10^{-10}

For IM-D9, the numbers of cells relative to those for IM were 227% for AST, 315% for O-2As, 196%for PROLs and 99% for OL s for experimental series -I (figure 5A). In experimental series - II, IM-D9 cell numbers relative to IM were 132% for AS, 160% for O-2As, 120% for PROLs and 140% for OLs for experimental series -II (figure 5B). Table 2 summarizes the statistical probabilities.

3.2.4 Alterations in the Levels of OL Specific Marker Proteins

To determine the changes in the counts of intact OLs could be correlated to the changes in the levels of proteins specific to the OLs, Western blots were performed for protein extracts from cultures washed and placed in BM/IM/BM-D9/BM-Ds9/IM-D9 for 18 hours. Anti-MBP (OL marker), anti-PLP (OL marker) antibodies were used to determine the levels of the OL marker proteins.

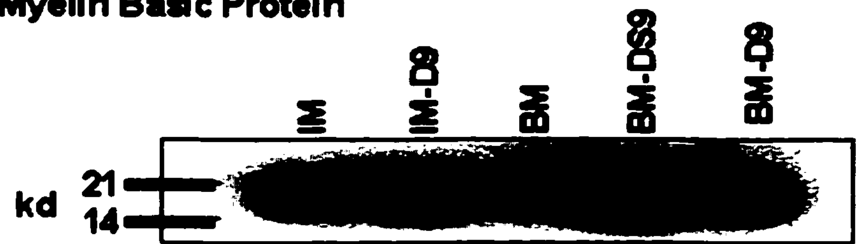
The anti-MBP antibody recognized two distinct isoforms of MBP at 21 and 14 kDa. As shown in the Western blot labeled myelin basic protein in figure 6, the immunodensity of the 21 and 14 kDa bands for the BM sample were reduced compared to the IM sample indicating that withdrawal resulted in a decrease in the level of MBP. Since equal amounts of total protein (protein concentrations were determined by spectrophotometry and the volume of protein from each treatment was determined for a predetermined protein concentration to be run in a gel) was loaded into each of the lanes, a decrease indicates a decreased proportion of MBP in the sample. The decrease could result from the net decrease in OLs relative to other cell types in samples as shown above. Hence the decrease would correspond, at least in part, to the greater proportional loss of

OLs than O-2A cells and AST cells shown above. It might also result from an induction of decreased expression of the MBP gene in response to insulin, IGF-I and serum withdrawal.

Probing of the blots for the second OL protein marker, PLP, revealed a visible 25 kDa band together with a faintly visible 30 kDa (Western blot labeled Proteolipid protein in figure 10). As in this example, there was no apparent change in the immunodensity for the anti-PLP antibody when the BM and IM samples were compared. A decrease in band density might be expected given the count data above that revealed a proportionally greater loss of OLs than O-2As and ASTs. A decrease in band density due to decreased numbers of OLs containing PLP may have been offset by an overall increase in PLP expression at the time of the samples.

The dramatic findings on the western blots were provided by the treatment of the IGF-I, insulin and serum withdrawn cells with 10^{-9} M (-)-deprenyl (lanes labeled BMD9) and 10^{-9} M (-)-desmethyldeprenyl, the major metabolite of (-)-deprenyl (lanes labeled BMDs9). For both of the OL marker proteins, including PLP, which did not show a decrease after IGF-I, insulin and serum withdrawal, treatment with either of the compounds resulted in a marked increase in immunodensity compared to BM. The increases are in keeping the increased survival of the OLs induced by (-)-deprenyl and (-)-desmethyldeprenyl (see below). For MBP and PLP, the band densities for BMD9 or BMDs9 exceed those for IM, suggesting that (-)-deprenyl and (-)-desmethyldeprenyl induced an increase in expression of the genes for the proteins.

Fig. 6. Effects of insulin, IGF-I and serum withdrawal and anti-apoptotic compounds on oligodendroglial protein levels. Total protein was extracted from the cells placed in BM, IM, BM-D9/BM-Ds9 or IM-D9 for 18 h after washing. Western blot analysis of the proteins was performed and the blots were probed with antibodies to MBP and PLP. MBP shows decreased immunodensity when placed in BM. (-)-Deprenyl as well as (-)-desmethyldeprenyl markedly increased the immunodensity of MBP and PLP. The data shown is representative of two separate experiments.



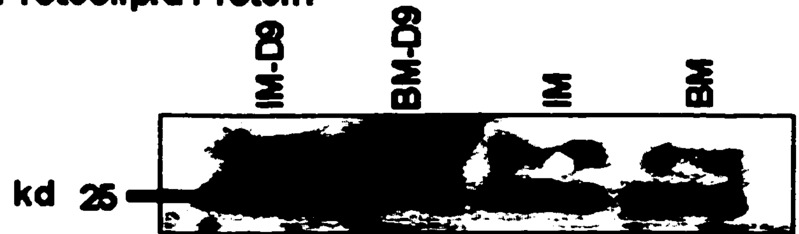


Figure 6

3.3 Oligodendroglial Cell Death on Withdrawal of Insulin, IGF-I and Serum

3.3.1 Morphological Changes

After washing and placement in BM, the web-like processes of OLs underwent fragmentation. Figure 7 show typical transmission (A1 and B1) interference contrast images (A2 and B2) of OLs at 18 h after washing that were immunoreacted for MBP. A1 and A2 are for cells that were washed and replaced in IM while B1 and B2 are for cells that were washed and placed in media only. B1 and B2 show the fragmentation of the OL processes with relative maintenance of cell body structure. The process fragmentation was noted increasing after 6 h following insulin, IGF-I and serum withdrawal.

3.3.2 Evidence For Apoptotic Degradation after Insulin, IGF-I and Serum Withdrawal

Electrophoresis gels for DNA extracted from the cells at 18 h after washing on 16 DIV showed DNA ladders with a period of about 180 bp for BM while electrophoresis for IM and BM-D9 did not show detectable laddering (figure 8A).

Fig. 7. Insulin, IGF-I and serum withdrawn oligodendrocytes display fragmented process morphology. Cells at 16 DIV were washed and replaced in IM or BM. Panels show transmitted light microscopic images of cells incubated in IM (A1) or in BM (B1) for 18h. Cells were subsequently fixed in 4% paraformaldehyde for 15 min and stained with an antibody to myelin basic protein (MBP). Panels (A2) and (B2) are interference contrast micrographs of MBP positive OLs. Note the extensively branched processes of OLs incubated in IM (A1, A2). Cells placed in BM show fragmented processes but display an intact cell body (B1, B2).

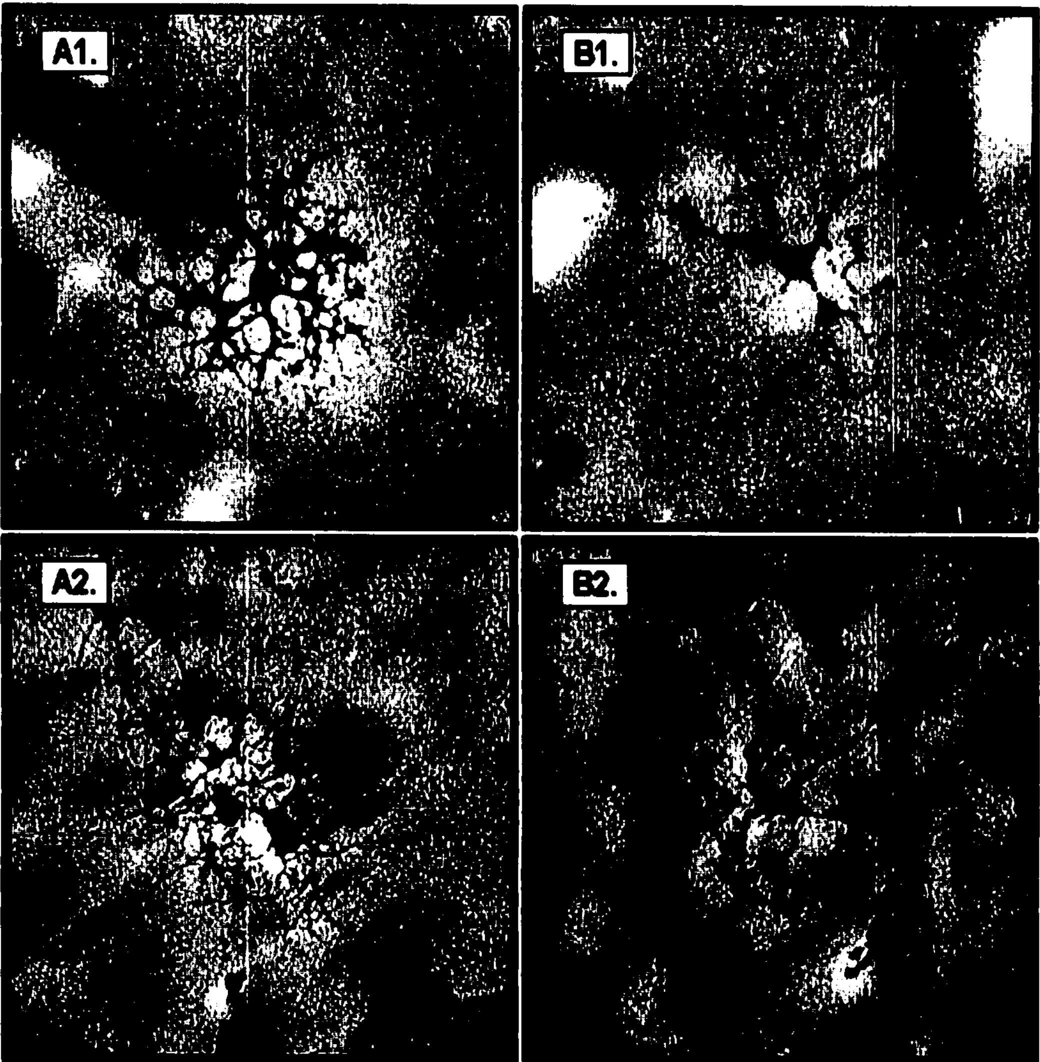


Figure 7

Fig. 8. Apoptosis of PROLs and OLs on insulin, IGF-I and serum withdrawal. Panel (A) shows the internucleosomal DNA degradation of PROLs and OLs. DNA "ladders" showing internucleosomal DNA digestion can be observed when cells were placed in BM for 18 h after washing. The ladder pattern is not seen when cells were incubated in IM or BM-D9. Panel (B) shows the nuclear DNA cleavage of apoptotic oligodendrocytes. Insulin and IGF-I withdrawn cells were stained for free 3' ends of cut DNA using the Apop Tag™ method (see Materials and Methods). Inset in panel (B) shows apoptotic OLs. The apoptotic nuclei typically show pyknosis and DNA fragmentation. Panel (C) shows an interference contrast micrograph of an apoptotic OL, as revealed by the Apop Tag™ method, displaying shrinkage of the cell body and fragmentation of processes. Panels (D1) and (D2) show fluorescence photomicrographs of PROLs and OLs deprived of insulin and IGF-I for 18 hours and stained with Hoechst 33258. Apoptotic cells show bright, condensed chromatin (open arrows) while non-apoptotic cells show nuclei with diffuse and granular pattern of staining (white arrows). Panel (D1) also shows an intensely fluorescent lobular structure (empty arrow) characteristic of an apoptotic body.

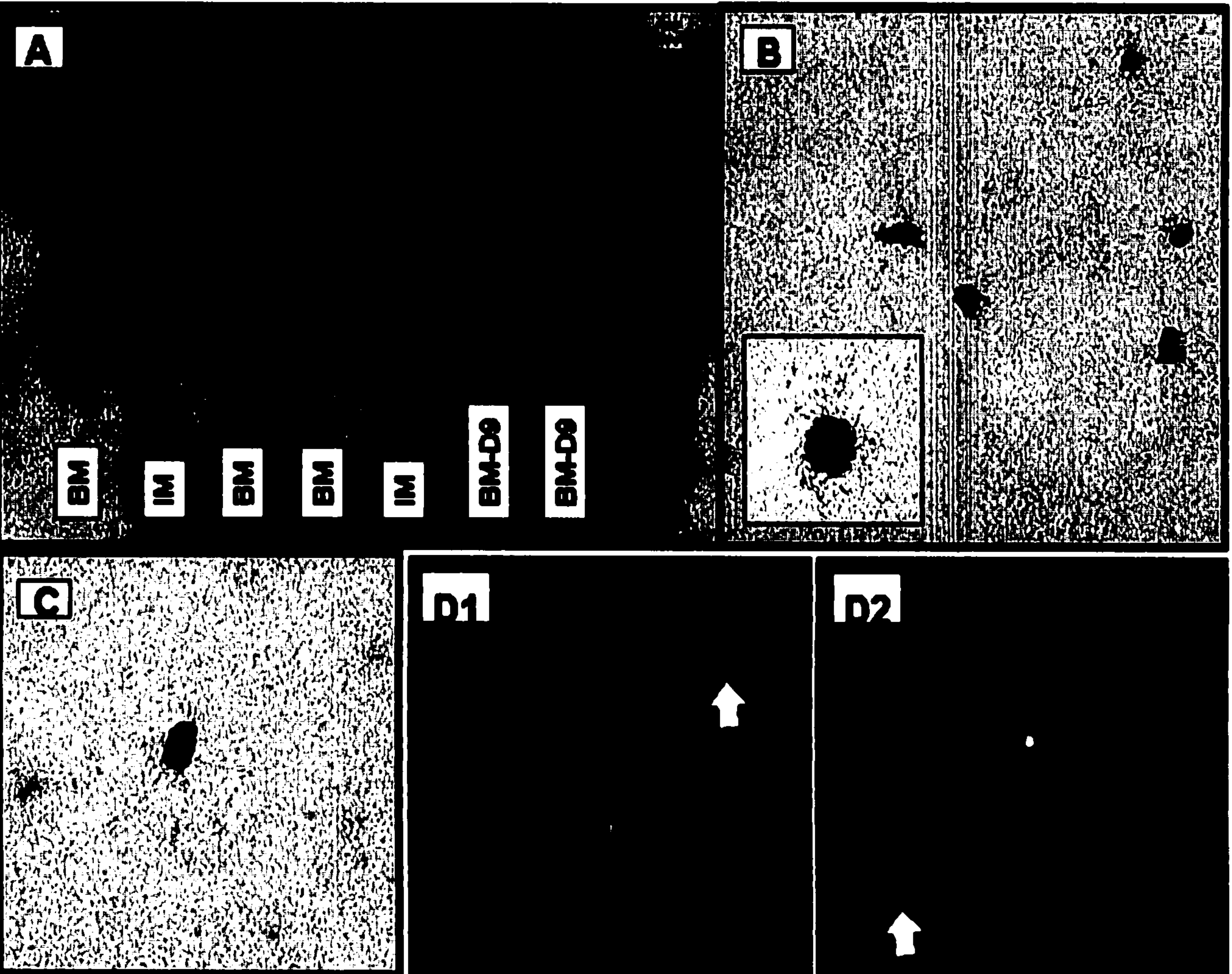


Figure8

Staining of the oligodendroglial cells with a supravital, bisbenzimide dye, Hoechst 33258 revealed nuclear chromatin condensation and typical apoptotic bodies (see fluorescence images in figure 7D1 and 7D2 for BM at 18 hours after washing, apoptotic bodies and condensed DNA are marked by open arrows while normally staining nuclei are marked by the white arrows). The Hoechst 33258 staining was identical to that which our laboratory found in neuronally differentiated PC12 cells after serum and NGF withdrawal (see Wadia *et al.*, 1998).

Similarly *in situ* end labeling (ISEL) using the ApopTag™ method revealed a proportion of nuclei in both BM and IM with evidence for DNA fragmentation as shown in the figure 8B and 8C (the images are for cells in BM at 18 hours after washing). Taken together the DNA ladders, chromatin staining and ISEL indicated that a significant proportion of the cells died by the process of apoptosis and the apoptosis was more evident after insulin, IGF-I and serum withdrawal than it was for cells supported by IGF-I and insulin.

This study therefore demonstrates apoptosis unequivocally in the differentiated cells, PROLs and OLs of the oligodendroglial lineage for the very first time using three independent methods including DNA gel electrophoresis, in situ end labeling and chromatin staining of apoptotic OLs.

3.3.3 Time Course and Magnitude of Apoptotic Nuclear Changes

Counts of ApopTag™ positive nuclei were obtained for 5 of the time points after washing to determine the time course of the appearance of apoptotic nuclear degradation

in the cultures as shown in figures 9A and 9B. Significant numbers of ApopTag™ positive nuclei were found for all time points for cells in IM (2.3% and 3.4% of total intact cell counts / mm^2 for the two series respectively). This finding indicated a baseline level of apoptosis in the cultures supported by IGF-I and insulin. A significant level of baseline apoptosis has been found in most neural culture systems (see Wadia *et al.*, 1998). The baseline level therefore must be taken into accord when judging the effects of agents that promote or reduce apoptosis. Addition of 10^{-9}M (-) -deprenyl to the IM did not significantly change the number of ApopTag™ positive nuclei at any time point in either experimental series.

Withdrawal of IGF-I, insulin and serum induced a gradual but marked increase in the number of ApopTag™ nuclei that reached a maximum at 18 hours after washing. Comparison of the values at each time point for BM and IM shows that the withdrawal of IGF-I, insulin and serum markedly increased the number of ApopTag™ nuclei above the baseline values found for IM. Addition of 10^{-9}M (-) -deprenyl to the base media, significantly decreased the numbers of ApopTag™ nuclei in both experimental series (see table 3 for statistical probabilities). These data clearly establish that the cultured oligodendroglial cells die by apoptosis after insulin, IGF-I and serum withdrawal and that (-) -deprenyl significantly reduces that apoptosis. It establishes that the increase in numbers of intact nuclei and methylene blue stained cells resulted at least in part from an anti-apoptotic action of (-) -deprenyl.

Fig. 9. Time course of apoptotic death of PROLs and OLs. Oligodendroglial cultures grown on coverslips at 16 DIV were incubated in IM/BM/BM-D9 or IM-D9 for 6, 12, 15, 18 and 24 h. Staining of fragmented nuclear DNA in OLs was performed using the Apop Tag™ method (see Materials and Methods). Apop Tag™ positive nuclei were counted in 25 randomly chosen low (100) power fields on triplicate coverslips. Data from experimental series I and II (A and B) are expressed as mean number ± SEM of apoptotic nuclei / mm^2. Note the occurrence of apoptotic nuclei after 6 h of trophic withdrawal, peaking at 18 h. (-) -Deprenyl reduced the incidence of apoptotic nuclei at all the time points after insulin, IGF-I and serum withdrawal. Data for each experimental series shown are derived from two separate experiments (n=50). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with IM, Mann-Whitney U test.

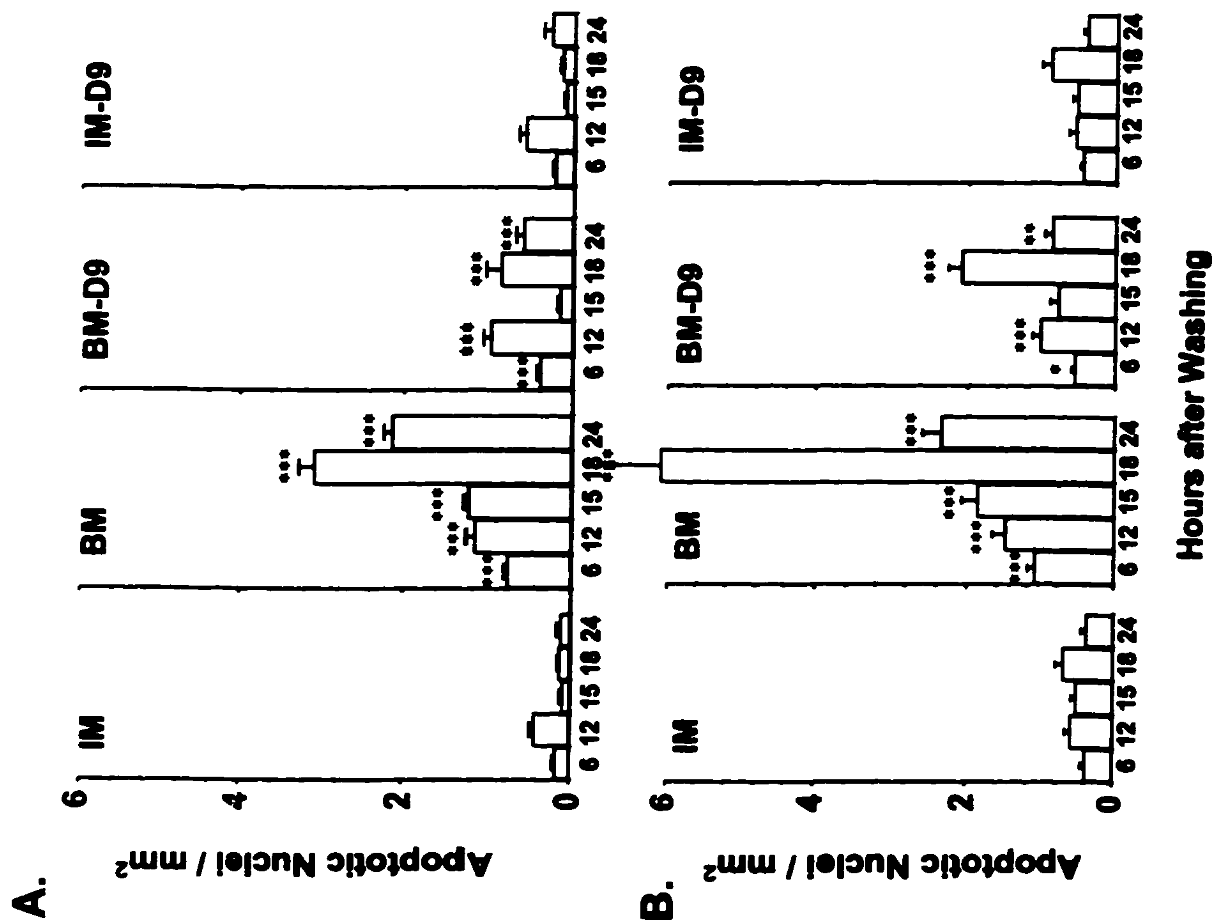


Figure 9

Table 3. Statistical analysis of data for time course of apoptosis of oligodendroglial cells

Experimental series - I

T- test for independent samples

Treatment	6h	12h	15h	18h	24h
IM-D9 / IM	4.8×10^{-1}	1.8×10^{-1}	1.0	8.4×10^{-1}	1.6×10^{-1}
BM / IM	1.9×10^{-7}	2.2×10^{-4}	1.0×10^{-13}	1.3×10^{-8}	1.2×10^{-5}
BM-D9 / IM	2.5×10^{-4}	5×10^{-6}	2.3×10^{-1}	1.3×10^{-4}	9.0×10^{-4}
BM-D9 / BM	8.4×10^{-4}	3.8×10^{-1}	1.7×10^{-12}	3.5×10^{-5}	5×10^{-6}

Mann - Whitney U Test

Treatment	6h	12h	15h	18h	24h
IM-D9 / IM	7.9×10^{-1}	4.9×10^{-1}	7.6×10^{-1}	9.9×10^{-1}	3.9×10^{-1}
BM / IM	4.6×10^{-5}	1.3×10^{-2}	6.2×10^{-13}	2.2×10^{-10}	1.2×10^{-5}
BM-D9 / IM	1.1×10^{-3}	2.0×10^{-5}	4.3×10^{-1}	3.0×10^{-3}	1.6×10^{-3}
BM-D9 / BM	1.3×10^{-2}	7.8×10^{-1}	7.9×10^{-12}	1.0×10^{-5}	7.3×10^{-4}

Experimental series - II

T- test for independent samples

Treatment	6h	12h	15h	18h	24h
IM-D9 / IM	3.5×10^{-1}	7.7×10^{-1}	7.8×10^{-2}	2.0×10^{-1}	7.4×10^{-1}
BM / IM	1.5×10^{-9}	1.7×10^{-5}	4.5×10^{-8}	7.5×10^{-10}	3.8×10^{-12}
BM-D9 / IM	2.2×10^{-2}	1.8×10^{-3}	3.6×10^{-2}	3.7×10^{-8}	2.0×10^{-4}
BM-D9 / BM	2.0×10^{-6}	3.6×10^{-2}	3.4×10^{-5}	2.4×10^{-8}	3.2×10^{-7}

Mann - Whitney U Test

Treatment	6h	12h	15h	18h	24h
IM-D9 / IM	3.4×10^{-1}	2.3×10^{-1}	8.3×10^{-1}	5.4×10^{-1}	7.9×10^{-1}
BM / IM	5.9×10^{-8}	3.0×10^{-3}	4.0×10^{-6}	2.1×10^{-10}	7.2×10^{-9}
BM-D9 / IM	2.8×10^{-2}	6.7×10^{-3}	2.4×10^{-1}	1.1×10^{-7}	3.4×10^{-3}
BM-D9 / BM	5.3×10^{-5}	8.2×10^{-2}	4.4×10^{-4}	2.9×10^{-5}	7.1×10^{-5}

3.4 (-)-Deprenyl but not (+)-Deprenyl Increases Oligodendroglial Survival

Studies in neuronally differentiated PC12 cells after serum and NGF withdrawal (Tatton *et al*., 1994) and immature facial neurons after axotomy (Ansari *et al*., 1993) showed that (-) -deprenyl decreased the apoptotic death of the cells while (+) -deprenyl did not have any effect on the survival of the cells. I examined the relative effects of the two enantiomers on oligodendroglial survival after IGF, insulin and serum withdrawal at three different concentrations (see figure 10). The (-) -enantiomer induced a concentration dependent increase in the numbers of intact nuclei with the greatest increase at 10^{-9} M. The gradual decrease in cell numbers with increasing concentration from 10^{-9} to 10^{-5} M is similar to that found for neuronally differentiated PC12 cells after serum and NGF withdrawal (Tatton *et al*., 1994). In that model, (-) -deprenyl was also most effective in reducing apoptosis at 10^{-9} M.

In the oligodendroglial cells, the (+) -enantiomer did not alter the numbers of intact nuclei from the values found for BM for any of the three concentrations tested. Accordingly, these results indicate that the anti-apoptotic action of (-)-deprenyl on the oligodendroglial cells is stereospecific.

Fig. 10. Concentration-survival relationship on treatment with S (+) and R (-) -deprenyl. After 16 DIV, cells were washed and incubated in BM or BM with 10^{-5} M, 10^{-7} M and 10^{-9} M, S (+) or R (-) -deprenyl for 24h. For controls, washed cells were replaced in IM. Cells were treated in quadruplicates (n=16). Intact nuclei were counted using a hemocytometer after Zap-oglobin lysis (see Materials and Methods). Data shown are mean ± SEM of intact nuclei relative to IM for two separate experiments. $***P < 0.001$, $**P < 0.01$, compared with BM, Mann-Whitney U test.

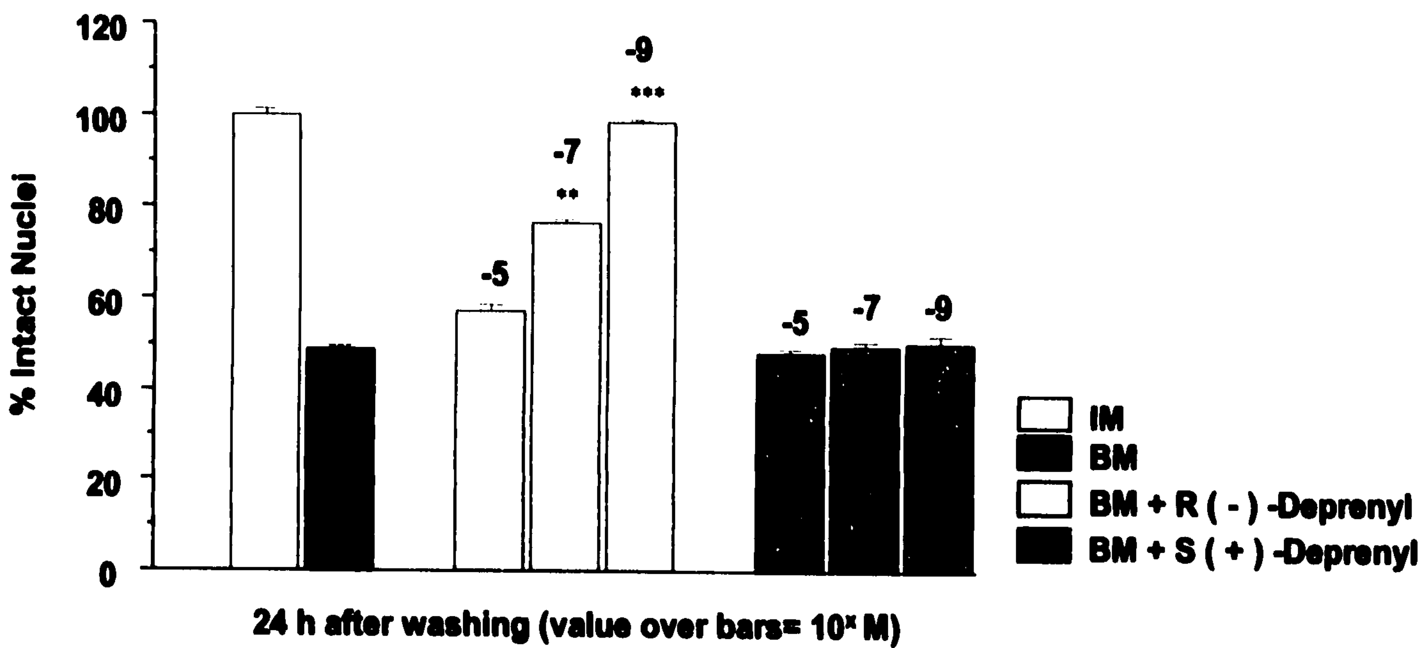


Figure 10

3.5 (-) -Desmethyldeprenyl, a Major Metabolite of (-) -Deprenyl is Responsible for the Reduction in Oligodendroglial Apoptosis

(-) -Deprenyl on oral administration is rapidly metabolized in the liver and the gastrointestinal tract by cytochrome P450 enzymes to three principal metabolites, (-) -desmethyldeprenyl, (-) -methamphetamine and (-) -amphetamine (Heinonen *et al.*, 1994; Rohatagi *et al.*, 1997a; Rohatagi *et al.*, 1997b). Experiments were carried out to determine the effects of the principal metabolites of (-) -deprenyl on oligodendroglial survival.

Withdrawal of insulin, IGF-I and serum for 24 hours led to intact nuclear counts in BM decreasing to 48.7% compared to those replaced into IM (compare white bars in the groups of bars labeled IM and BM in figure 11). As shown in the experiments presented above, washing and placement of the cells in media without IGF-I and insulin, addition of 10^{-9} M (-)-deprenyl increased the counts of intact nuclei by 194% compared to those for BM (compare the white and black bars in the BM group of figure 11). Treatment of cultures with 10^{-9} M (-)-desmethyldeprenyl induced an almost identical 197% increase in the counts (compare the white and gray bars in the BM group of figure 11). Controls of adding (-)-deprenyl or (-)-desmethyldeprenyl to cells supported by IGF-I and insulin resulted in the numbers of intact nuclei that were 107% and 103% relative to IM (group labeled IM in figure 11).

In contrast to (-)-desmethyldeprenyl, (-)-methamphetamine (MA) or (-)-amphetamine (AM) did alter the counts of intact nuclei. Each was tested at three concentrations of 10^{-5}, 10^{-7} and 10^{-9} M (compare white bars in the groups labeled

BM+MA and BM+AM to the white bar in the BM group of figure 11). The statistical probabilities are shown in Table 5. These data suggest that (-)-desmethyldeprenyl but not (-)-methamphetamine or (-)-amphetamine may mediate the anti-apoptotic actions of (-)-deprenyl on the oligodendroglial cells. It has previously been shown that (-)-deprenyl and (-)-desmethyldeprenyl are similarly effective in reducing apoptosis in primary cultures of rat dopaminergic cells after exposure to excitotoxicity (Mytilineou *et al.*, 1997) but it is not known whether one or both of the agents mediate the anti-apoptotic effect. I used blockade of P450 enzyme metabolism of (-)-deprenyl to determine whether one or both mediated the anti-apoptosis.

Three general inhibitors of P450 enzymes, proadifen, metapyrone and piperonyl butoxide, were used to block the P450 enzyme dependent metabolism of (-) -deprenyl into its metabolites. Addition of proadifen at 2.5, 10 and 25μM with 10^{-9} M (-) -deprenyl resulted in a decrease in the number of intact nuclei to 144%, 109% and 91% respectively when compared to IM (compare the black bars in the groups labeled BM+ Proad to that in the BM group in figure 11). Importantly, the three concentrations of proadifen did alter the values for BM only (compare the white bars in the groups labeled BM+ Proad to that in the BM group in figure 11). Furthermore, the three concentrations of proadifen did not reduce the increase in intact nuclei induced by 10^{-9} M (-) -desmethyldeprenyl (compare the gray bars in the groups labeled BM+ Proad to that in the BM group in figure 11). The two other cytochrome P450 enzyme blockers, metapyrone at 50 μM and piperonyl butoxide at 100μM had similar actions to proadifen (groups labeled BM+ Meta and BM+P.But. in figure 11).

Fig. 11. Effects of general cytochrome P450 blockers on (-)-deprenyl and (-)-desmethyldeprenyl induced changes in OL cell survival. OLs grown on glass coverslips were incubated in BM supplemented with 10^{-9} M (-)-deprenyl or (-)-desmethyldeprenyl and treated with the general cytochrome P450 blockers proadifen, metapyrone, or piperonyl butoxide for 24h in quadruplicates. Cells were also treated with (-)-methamphetamine or (-)-amphetamine alone in BM or with 10^{-9} M (-)-deprenyl or (-)-desmethyldeprenyl. Concentrations of the P450 blockers as well as of (-)-methamphetamine or (-)-amphetamine are indicated above the respective bars. Intact nuclei were counted on a haemocytometer following Zap-oglobin lysis. Control cells were washed and incubated in IM. Data shown represent the mean percentage of intact nuclei ± SEM relative to IM for two separate experiments (n=16-20). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with BM, Mann-Whitney U test.

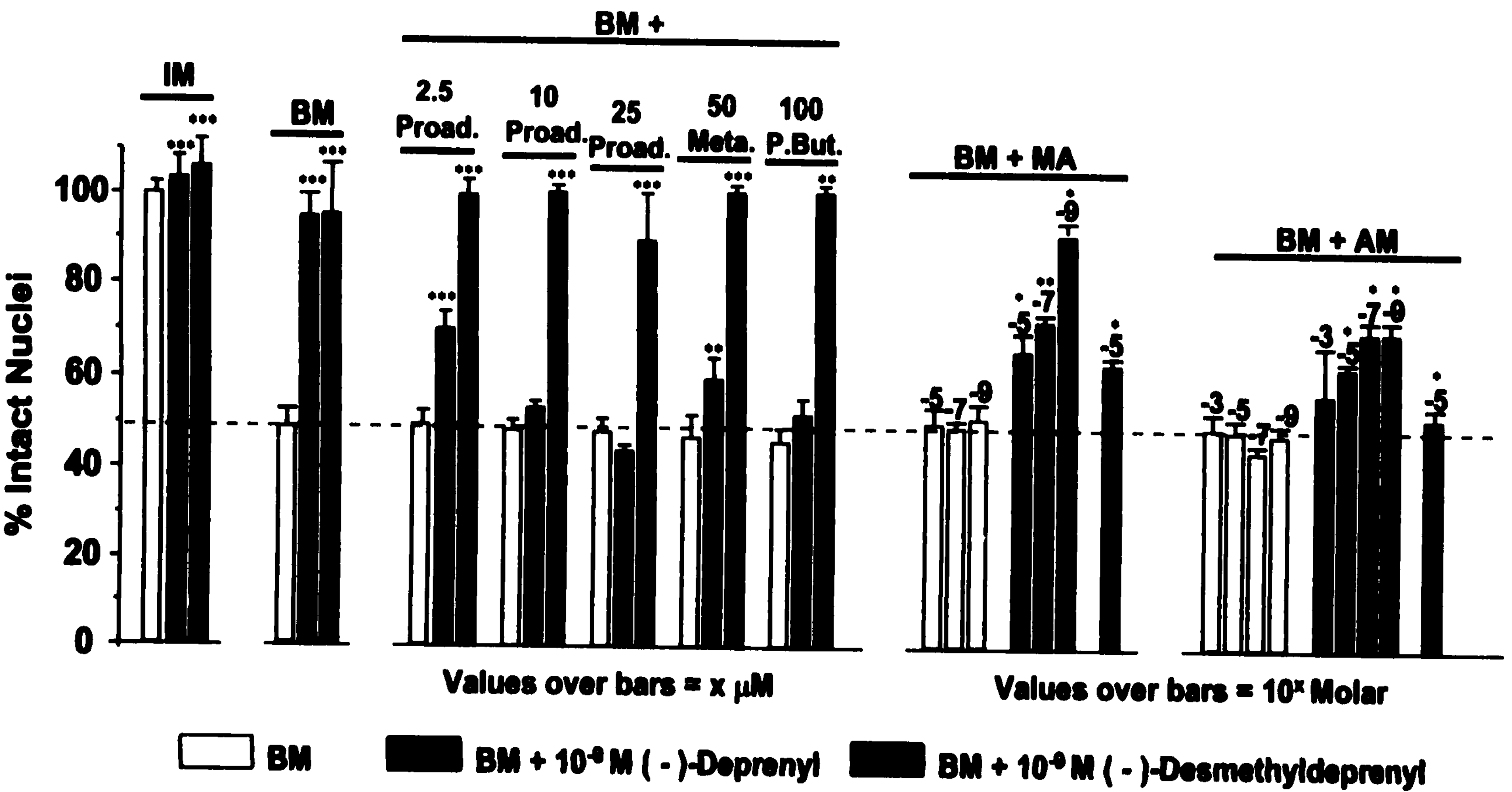


Figure 11

The statistical probabilities for these data are indicated in Table 4. The results obtained show that the general cytochrome P450 blockers completely eliminated the survival enhancing effect of (-)-deprenyl but did not alter the capacity of (-)-desmethyldeprenyl to increase the survival of oligodendroglial cells. Hence the anti-apoptotic effect of (-)-deprenyl on the oligodendroglial cells requires its metabolism to (-)-desmethyldeprenyl.

3.6 (-) -Methamphetamine and (-) -Amphetamine Competitively Reduce the Capacity of (-) -Desmethyldeprenyl to Reduce Oligodendroglial Apoptosis

Studies of facial motoneuron survival after axotomy in mice showed that treatment with (-)-methamphetamine and (-)-amphetamine reduced the effectiveness of (-)-deprenyl in improving the survival of the motoneurons (Oh *et al.*, 1994). The basis for the reduction in effectiveness is not known. I therefore undertook competition experiments to determine whether either of the metabolites reduced the capacity of (-)-deprenyl or (-)-desmethyldeprenyl to increase oligodendroglial survival after insulin, IGF-I and serum withdrawal.

Methamphetamine when used in 10^{-5} M, 10^{-7} M and 10^{-9} M concentrations in combination with 10^{-9} M (-) -deprenyl significantly reduced the increase in numbers of intact nuclei induced by (-)-deprenyl in a graded concentration dependent manner (compare the black bars in the BM+MA group with the black bar in the BM group of figure 11) and 10^{-5} M (-)-methamphetamine had a similar competitive action on 10^{-9} M (-)-desmethyldeprenyl (compare the gray bar in the BM+MA group with the black bar

in the BM group of figure 11).

Similarly (-) -amphetamine was used at 10^{-3} M, 10^{-5} M, 10^{-7} M and 10^{-9} M concentrations in combination with 10^{-9} M (-) -deprenyl or 10^{-9} M (-)-desmethyldeprenyl (compare the black and gray bars in the group labeled BM+AM with the black and gray bar in the BM group). Although, the addition of (-)-amphetamine markedly reduced the capacity of (-)-deprenyl to increase the survival of the oligodendroglial cells after insulin, IGF-I and serum withdrawal, the concentration dependent survival reduction was not as evident as with (-)-methamphetamine, in part due to greater sample to sample variation (see table 5 for statistical probabilities). (-)-Amphetamine at 10^{-5} M completely eliminated the capacity of 10^{-9} M (-)-desmethyldeprenyl to increase the survival of the oligodendroglial cells.

These data suggest that the tissue levels of (-)-methamphetamine and/or (-)-amphetamine can be major factors in determining the effectiveness of (-)-deprenyl or (-)-desmethyldeprenyl in reducing oligodendroglial apoptosis. As shown above, the competition does not appear to reflect a pro-apoptotic effect of (-)-methamphetamine or (-)-amphetamine but rather seems to depend on an interference with the mechanism(s) underlying the anti-apoptosis by (-)-desmethyldeprenyl.

Table 4. Statistical analysis of data for intact nuclear counts after drug treatments

Treatment	T-test for independent samples Intact nuclear counts	Mann-Whitney U test Intact nuclear counts
BM / IM	7.5×10^{-29}	6.3×10^{-8}
IM + (-)-Depr / BM	0.0	2.7×10^{-10}
IM + (-)-Desmeth / BM	3.4×10^{-26}	3.0×10^{-7}
BM + (-)-Depr / BM	0.0	3.5×10^{-7}
BM + (-)-Desmeth / BM	5.8×10^{-21}	2.7×10^{-10}
BM + 2.5 μM Proad / BM	3.3×10^{-1}	4.4×10^{-1}
BM + 10 μM Proad / BM	1.0	3.7×10^{-1}
BM + 25 μM Proad / BM	8.3×10^{-1}	8.8×10^{-1}
BM + 50 μM Metyr / BM	9.6×10^{-2}	1.1×10^{-1}
BM +100 μM Pip.But / BM	1.3×10^{-2}	3.1×10^{-2}
BM + 2.5 μM Proad + (-)-Depr / BM	2.7×10^{-9}	1.6×10^{-4}
BM + 2.5 μM Proad + (-)-Desmeth / BM	7.8×10^{-11}	1.6×10^{-4}
BM + 10 μM Proad + (-)-Depr / BM	5.3×10^{-4}	4.0×10^{-3}
BM + 10 μM Proad + (-)-Desmeth / BM	2.3×10^{-10}	4.0×10^{-3}
BM + 25 μM Proad + (-)-Depr / BM	4.9×10^{-1}	5.7×10^{-1}
BM + 25 μM Proad + (-)-Desmeth / BM	9.3×10^{-10}	4.0×10^{-3}
BM + 50 μM Metyr + (-)-Depr / BM	5.0×10^{-6}	1.6×10^{-4}
BM + 50 μM Metyr + (-)-Desmeth / BM	2.3×10^{-10}	4.0×10^{-3}
BM + 100μM Pip.But + (-)-Depr / BM	1.0×10^{-1}	1.0×10^{-1}
BM + 100μM Pip.But + (-)-Desmeth / BM	2.3×10^{-10}	4.0×10^{-3}

Table 5. Statistical analysis of data for intact nuclear counts after methamphetamine and amphetamine treatments

Treatment	T-test for independent samples Intact nuclear counts	Mann-Whitney U test Intact nuclear counts
BM + (-)-Meth 10^{-5}M / BM	7.4×10^{-1}	1.07
BM + (-)-Meth 10^{-7}M / BM	1.0	1.0
BM + (-)-Meth 10^{-9}M / BM	3.2×10^{-1}	4.9×10^{-1}
BM + (-)-Meth 10^{-5}M + (-) -Depr / BM	1.0×10^{-6}	4.0×10^{-3}
BM + (-)-Meth 10^{-5}M + (-) -Desmeth / BM	6.5×10^{-5}	2.9×10^{-2}
BM + (-)-Meth 10^{-7}M + (-) -Depr / BM	5.7×10^{-7}	2.9×10^{-2}
BM + (-)-Meth 10^{-9}M + (-) -Depr / BM	5.1×10^{-8}	2.9×10^{-2}
BM + (-)-Amph 10^{-3}M / BM	2.6×10^{-1}	2.8×10^{-1}
BM + (-)-Amph 10^{-5}M / BM	1.0	2.6×10^{-1}
BM + (-)-Amph 10^{-7}M / BM	2.9×10^{-3}	4.0×10^{-3}
BM + (-)-Amph 10^{-9}M / BM	2.0×10^{-1}	2.8×10^{-1}
BM + (-)-Amph 10^{-3}M + (-) -Depr / BM	1.5×10^{-1}	5.0×10^{-1}
BM + (-)-Amph 10^{-5}M + (-) -Depr / BM	1.0×10^{-1}	4.0×10^{-3}
BM + (-)-Amph 10^{-5}M + (-) -Desmeth / BM	1.7×10^{-2}	2.8×10^{-2}
BM + (-)-Amph 10^{-7}M + (-) -Depr / BM	3.0×10^{-7}	4.0×10^{-3}
BM + (-)-Amph 10^{-9}M + (-) -Depr / BM	3.0×10^{-7}	4.0×10^{-3}

3.7 (-) -Desmethyldeprenyl Increases the Survival of Oligodendroglial Cells by A New Protein Synthesis Dependent Mechanism

To determine if the increases in survival effected by (-) -desmethyldeprenyl were dependent on transcription or translation in a similar manner to that previously shown for the neuronally differentiated PC12 cells (Tatton *et al.*, 1994), the oligodendroglial cultures were treated with a transcriptional blocker, actinomycin and a translational blocker, cycloheximide. Actinomycin was used at 1, 2.5 and 5 μg / ml while cycloheximide was used at 1, 5, 10, and 50 μg/ml concentrations. Neither agent altered the numbers of intact nuclei for cells that had undergone withdrawal of insulin, IGF-I and serum (compare the white bar in the BM group to the white bars in the BM + Cycloheximide and the BM + Actinomycin groups in figure 12). Similar to undifferentiated PC12 cells after serum withdrawal (Rukenstein *et al.*, 1991) and neuronally differentiated PC12 cells after serum and NGF withdrawal (Tatton *et al.*, 1994), this data indicates that apoptosis in the oligodendroglial cells is independent of a requirement for new protein synthesis and proceeds through signaling by constitutive proteins.

In contrast to their action on apoptosis itself, both agents reduced the capacity of 10^{-9} M (-)-desmethyldeprenyl to increase the number of the oligodendroglial cells after insulin, IGF-I and serum withdrawal. The reduction in the capacity of (-)-desmethyldeprenyl to increase cell numbers was concentration dependent and induced a complete loss of the (-)desmethyldeprenyl induced survival at concentrations between 10 and 50 μg/ml for cycloheximide and 2.5 and 5.0 μg/ml for actinomycin D.

See tables 6 and 7 for statistical probabilities.

Fig. 12. Reduction of oligodendroglial apoptosis by (-)-desmethyldeprenyl is protein synthesis dependent. After 16 DIV, cells were washed and incubated in BM, BM-Ds9 or BM-Ds9 plus the translation or transcription blockers cycloheximide or actinomycin respectively in quadruplicates. Concentrations of cycloheximide and actinomycin are indicated above the respective bars. Intact nuclei were counted on a hemocytometer following Zap-oglobin lysis. Control cells were washed and incubated in IM. Data shown represent the mean ± SEM (n=16) of intact nuclei relative to IM for two separate experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with BM, Mann-Whitney U test.

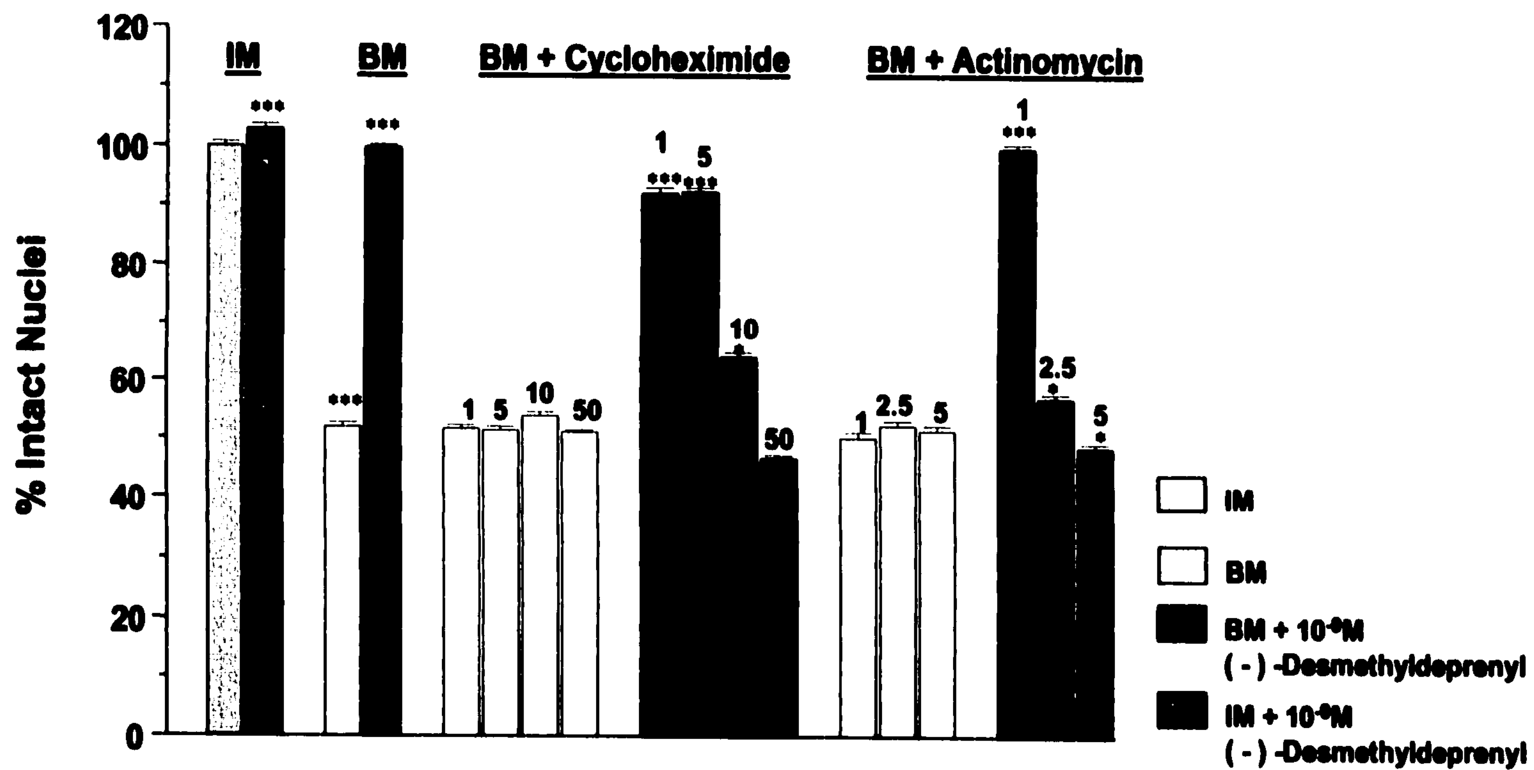


Figure 12

Table 6. Statistical analysis of data for intact nuclear counts for cycloheximide treatment

Treatment	T-test for independent samples Intact nuclear counts	Mann-Whitney U test Intact nuclear counts
BM / IM	2.5×10^{-16}	7.7×10^{-4}
BM + (-)-Desmeth / BM	1.6×10^{-17}	7.7×10^{-4}
IM + (-)-Desmeth / BM	3.7×10^{-17}	7.2×10^{-4}
BM + 1µM CHX / BM	7.4×10^{-1}	8.7×10^{-1}
BM + 5µM CHX / BM	5.3×10^{-1}	6.2×10^{-1}
BM + 10µMCHX / BM	8.0×10^{-2}	1.1×10^{-1}
BM + 50µMCHX / BM	3.0×10^{-1}	5.2×10^{-1}
BM + 1µM CHX + Desmeth. / BM	4.2×10^{-14}	7.7×10^{-4}
BM + 5µMCHX + Desmeth. / BM	7.9×10^{-16}	7.7×10^{-4}
BM + 10µMCHX + Desmeth. / BM	2.4×10^{-8}	7.7×10^{-4}
BM + 50µMCHX + Desmeth. / BM	4.2×10^{-5}	7.7×10^{-5}

Table 7. Statistical analysis of data for intact nuclear counts for actinomycin treatment

Treatment	T-test for independent samples Intact nuclear counts	Mann-Whitney U test Intact nuclear counts
BM / IM	2.5×10^{-16}	7.7×10^{-4}
BM + (-)-Desmeth / BM	1.6×10^{-17}	7.7×10^{-4}
IM + (-)-Desmeth / BM	3.7×10^{-17}	7.2×10^{-4}
BM + 1μg/ml ACT / BM	1.3×10^{-1}	1.5×10^{-1}
BM + 2.5μg/ml ACT / BM	7.7×10^{-1}	7.9×10^{-1}
BM + 5μg/ml ACT / BM	6.0×10^{-1}	5.2×10^{-1}
BM + 1μg/ml ACT + Desmeth. / BM	4.0×10^{-17}	7.7×10^{-4}
BM + 2.5μg/ml ACT + Desmeth. / BM	3.6×10^{-4}	3.2×10^{-3}
BM + 5μg/ml ACT + Desmeth. / BM	3.9×10^{-3}	1.1×10^{-2}

Those concentrations are within the range that reduces new protein synthesis to 10% or less in other cell culture systems (see Rukenstein *et al.*, 1991; Tatton *et al.*, 1994). Accordingly, (-)-desmethyldeprenyl appears to require new protein synthesis to reduce apoptosis caused by insulin, IGF-I and serum withdrawal in oligodendroglial cells. The interpretation of a need for new protein synthesis is strengthened by the finding that both a translational and a transcriptional inhibitor block the increased survival induced by desmethyldeprenyl.

3.8 Mitochondrial Membrane Potential In Oligodendroglial Apoptosis

Studies in primary cultures of cerebellar neurons (Paterson *et al.*, 1998) and in neuronally differentiated PC12 cells (Wadia *et al.*, 1998) have shown that (-)-deprenyl prevents the decrease in $\Delta\Psi_M$ that occurs early in many forms of apoptosis and results in opening of the PTP (see details above). Measurements of mitochondrial CMTMR fluorescence intensities imaged with laser confocal microscopy were used to estimate the $\Delta\Psi_M$ in the oligodendroglial cells. CMTMR has been compared to other mitochondrial potentiometric dyes and has been shown to offer a reliable estimate of $\Delta\Psi_M$ in individual mitochondria *in situ* (see Wadia *et al.*, 1998). $\Delta\Psi_M$ was measured at 18 hours after washing, the time point at which maximum numbers of apoptotic nuclei were found to be present in the oligodendroglial cells (see above).

The distributions of mitochondrial CMTMR fluorescence for PROLs, OLs and ASTs in IM indicated that OL and PROL mitochondria have markedly higher levels of $\Delta\Psi_M$ than AST mitochondria (see the upper row of distributions in figure 13, typical

CMTMR laser confocal microscope images for each type under the corresponding treatment conditions are superimposed on each distribution in figures 13-16). The shape of the distributions indicates that OLs and PROLs have a high proportion of mitochondria with relatively high level $\Delta\Psi_M$. Withdrawal of IGF-I, insulin and serum shifted with mitochondrial CMTMR distributions to significantly lower values for the ASTs, PROLs and OLs (see the lower row of distributions in figure 13). The relative decreases were particularly marked for the PROL and OL mitochondria. These finding indicate that apoptosis induced in PROLs and OLs by insulin, IGF-I and serum withdrawal involves a decrease in $\Delta\Psi_M$ and likely is mitochondrially dependent apoptosis (see above).

Treatment with 10^{-9} M (-) -deprenyl (see the upper row of distributions in figure 14) and 10^{-9} M (-) -desmethyldeprenyl (see the lower row of distributions in figure 14) shifted the CMTMR fluorescence distributions for mitochondria in ASTs, PROLs and OLs to levels that were not significantly different to those found for IM (see Table 8). Similar to cultured cerebellar neurons (Paterson *et al.*, 1998) and neuronally differentiated PC12 cells (Paterson *et al.*, 1998), (-)-deprenyl prevented the decreases in PROLs and OLs which may account for its capacity to reduce apoptosis in the cells. This is the first study to show that (-)-desmethyldeprenyl shares the capacity of (-)-deprenyl to maintain $\Delta\Psi_M$ in cells that have sustained an insult that leads to apoptosis, in this case insulin, IGF-I and serum withdrawal.

Fig. 13. Effects of insulin, IGF-I and serum withdrawal on mitochondrial membrane potential ($\Delta\Psi_M$) of oligodendroglial lineage cells. Cells grown on glass coverslips were incubated in IM (A) or BM (B) for 18h, and then incubated with the potentiometric dye CMTMR (138 nM) for 15 min at 37°C before fixation with 4% paraformaldehyde on ice for 30 min. Confocal images with constant power and pinhole aperture settings were obtained of each cell type, ASTs, PROLs, OLs. A minimum of 20 images were obtained for each cell type. Using the image acquisition and analysis software Metamorph™, 2 regions within an individual mitochondrion were selected and the fluorescence intensity was measured on a scale of 0-255. No fewer than 20 such regions were measured from each cell. Fluorescence intensity measurements were obtained from 20 cells / treatment. The distribution of CMTMR fluorescence intensity observed in each cell type and the mean intensity ± SEM are shown. Note the higher percentage of mitochondria in ASTs with low CMTMR fluorescence intensity compared to PROLs and OLs grown in IM. Also note the shift to the left in the distribution of CMTMR fluorescence intensity in each cell type when incubated in BM, indicating a reduction in $\Delta\Psi_M$. OLs and PROLs have a high proportion of mitochondria with relatively high level $\Delta\Psi_M$.

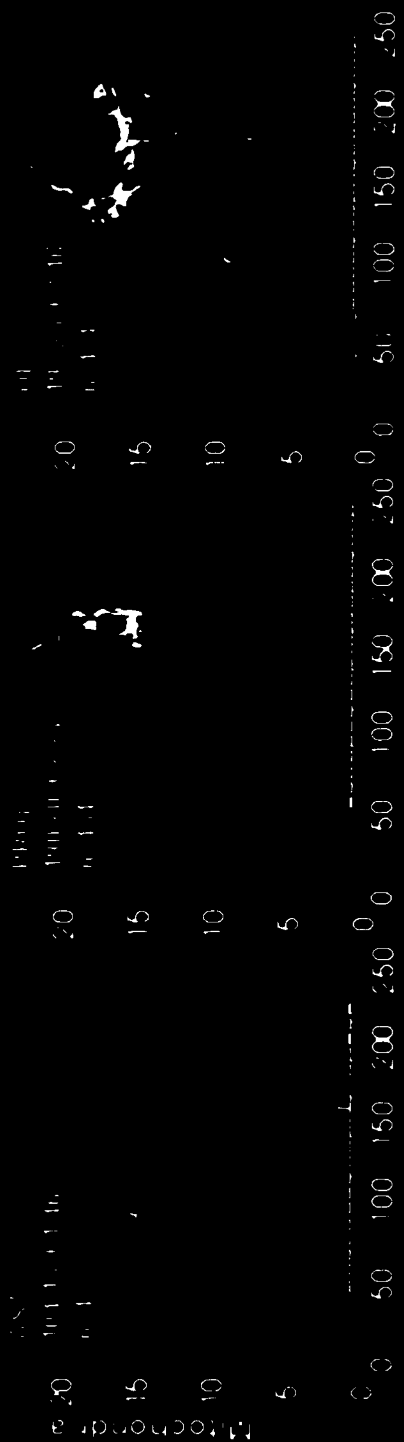
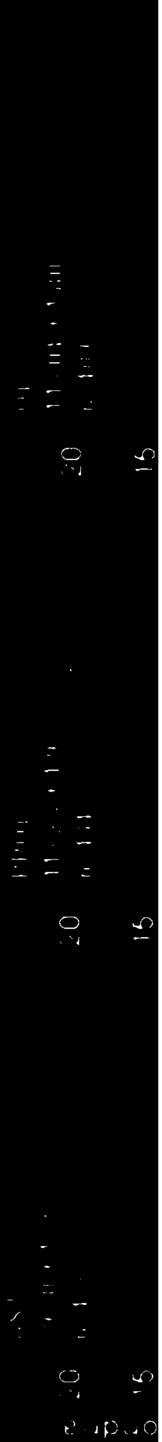
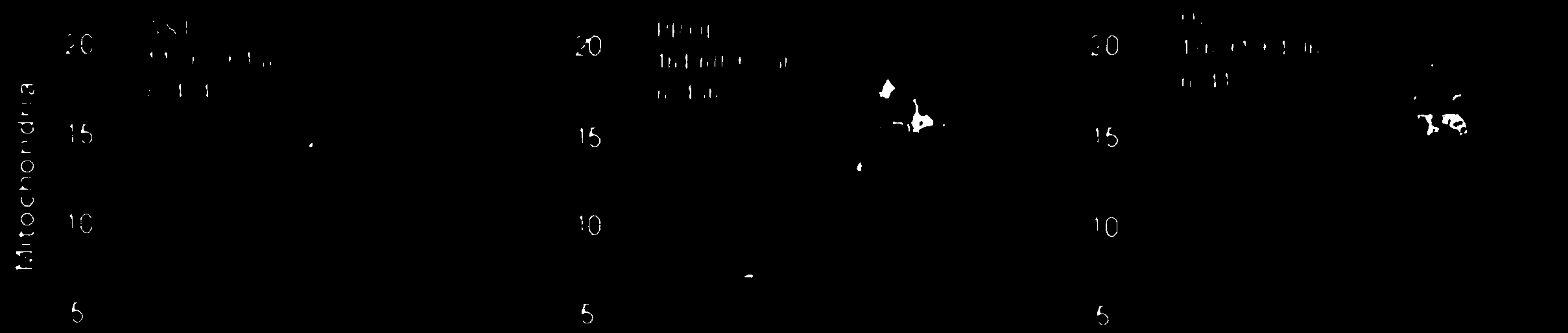
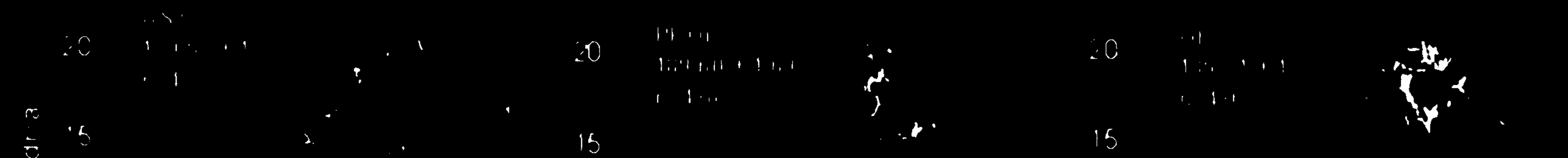



Fig. 14. Effects of (-) -deprenyl and (-) -desmethyldeprenyl on $\Delta\Psi_M$ of oligodendroglial lineage cells. Cells grown on glass coverslips were incubated in BM-D9 (A) or BM-Ds9 (B) for 18h and stained with CMTMR as before followed by fixation with 4% paraformaldehyde. Confocal images were obtained with constant power and pinhole aperture settings of ASTs, PROLs and ASTs. CMTMR fluorescence intensity values were obtained as described using the Metamorph™ image analysis software from 20 individual mitochondria within 20 cells. Values are the mean ± SEM intensity of CMTMR fluorescence. Also shown are frequency distributions of mitochondrial CMTMR fluorescence in each treatment. Note that both (-) -deprenyl and (-) -desmethyldeprenyl prevented the decrease in $\Delta\Psi_M$.


A BMD9
Mitochondria
20
15
10
5
0
0 50 100 150 200 250
B BMDss9



Experiments in which proadifen (10μM), metapyrone (50μM) or piperonyl butoxide (100μM) were added to IGF-I,insulin and serum withdrawn cells that were treated with 10^{-9} M (-)-deprenyl showed that prevention of (-)-deprenyl metabolism by cytochrome P450 inhibition blocked the increases in $\Delta\Psi_M$ induced by (-)-deprenyl on OL mitochondria (see the upper row of distributions in figure 15). Similar experiments using the three P450 inhibitors with (-)-desmethyldeprenyl, rather than (-)-deprenyl, showed prevention of (-)-deprenyl metabolism did not compromise the capacity of (-)-desmethyldeprenyl to maintain $\Delta\Psi_M$ in OL mitochondria after insulin, IGF-I and serum withdrawal (see the lower row of distributions in figure 15). These findings appear to correspond to those above in which the three P450 inhibitors blocked the capacity of (-)-deprenyl to increase oligodendroglial survival but did not affect the capacity of (-)-desmethyldeprenyl to increase the survival.

In a final series of experiments, CMTMR mitochondrial fluorescence was measured in IGF-I , insulin and serum withdrawn OL mitochondria treated with 10^{-9} M (-)-deprenyl in combination with 10^{-5} M (-)-methamphetamine or 10^{-5} M (-)-amphetamine (see upper row of distributions in figure 16). Similar experiments were carried out with 10^{-9} M (-)-desmethyldeprenyl rather than (-)-deprenyl (see lower row of distributions in figure 16). These data show that (-)-methamphetamine and (-)-amphetamine competition reduce the capacity of (-)-deprenyl and (-)-desmethyldeprenyl to maintain $\Delta\Psi_M$ in OL mitochondria after insulin, IGF-I and serum withdrawal. These findings appear to correspond to the findings presented above showing that (-)-methamphetamine and (-)-amphetamine competition reduces the capacity of

(-)-deprenyl and (-)-desmethyldeprenyl to increase the survival of oligodendroglial cells after insulin, IGF-I and serum withdrawal.

Fig. 15. Effects of general cytochrome P450 blockers on (-)-deprenyl and (-)-desmethyldeprenyl induced changes in $\Delta\Psi_M$. OLs grown on glass coverslips were incubated in BM supplemented with 10^{-9} M (-)-deprenyl or (-)-desmethyldeprenyl and treated with the general cytochrome P450 blockers proadifen, metapyrone, or piperonyl butoxide for 18h. At the end of the treatment period cells were incubated with CMTMR as before and fixed in 4% paraformaldehyde. Confocal images were obtained using constant power and pinhole aperture settings and CMTMR intensity was measured using the Metamorph™ image analysis software from 20 individual mitochondria within 20 cells per treatment. Values represent the mean ± SEM intensity of CMTMR fluorescence. The frequency distributions of mitochondrial CMTMR fluorescence are also shown. Note that treatment with general cytochrome P450 blockers prevented the maintenance of $\Delta\Psi_M$ provided by (-) -deprenyl but not by (-) -desmethyldeprenyl, indicating that metabolism of (-) -deprenyl to (-) -desmethyldeprenyl is necessary for its protective effects.

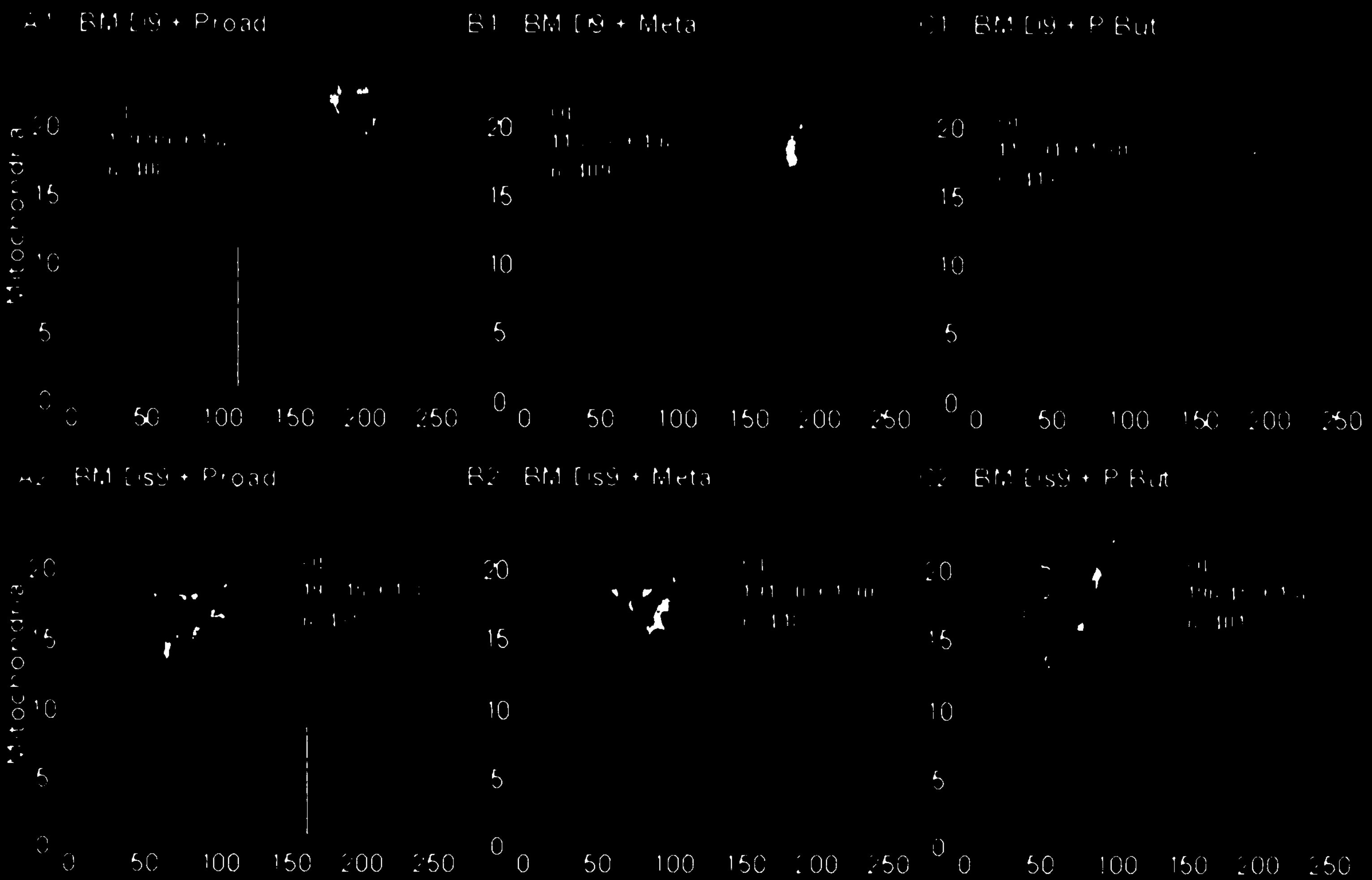
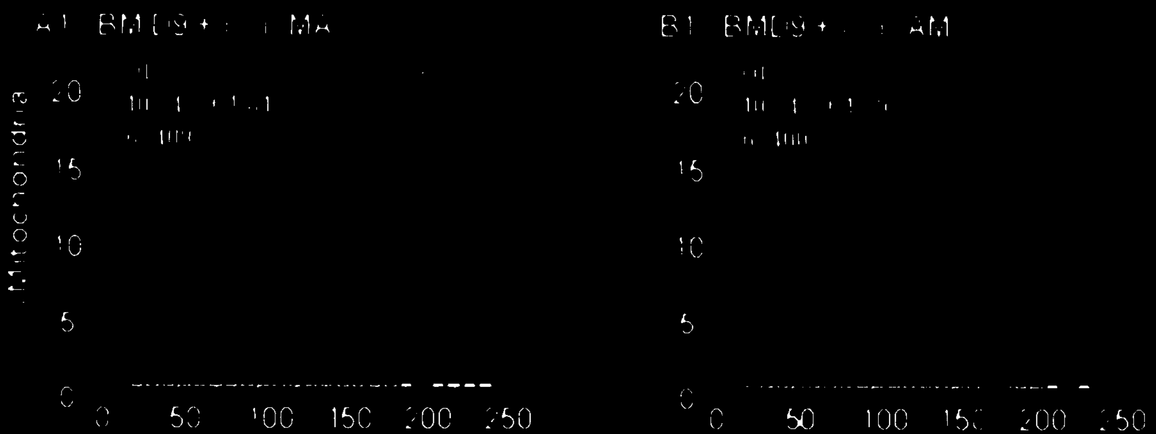


Figure 15

Fig. 16. Effects of (-) -methamphetamine and (-) -amphetamine on (-) -deprenyl and (-) -desmethyldeprenyl induced changes in $\Delta\Psi_M$ of oligodendroglial cells. OLs grown on glass coverslips were incubated in BM for 18h supplemented with 10^{-9} M (-) -deprenyl or (-) -desmethyldeprenyl and treated with 10^{-5} M (-) -methamphetamine or 10^{-5} M (-) -amphetamine. Cells were incubated with CMTMR as described and fixed in 4% paraformaldehyde. Confocal images were obtained using constant power and pinhole aperutre settings of a minimum of 20 cells / treatment. CMTMR fluorescence intensity was measured using the Metamorph™ image analysis software from a minimum of 20 individual mitochondria in each cell. Values represent the mean ± SEM intensity of CMTMR fluorescence. The frequency distributions of mitochondrial CMTMR fluorescence are also shown. Note that the addition of (-) -methamphetamine or (-) -amphetamine to cells incubated in BM supplemented with (-) -deprenyl or (-) -desmethyldeprenyl prevented the maintenance of $\Delta\Psi_M$ provided by both (-) -deprenyl and (-) -desmethyldeprenyl.



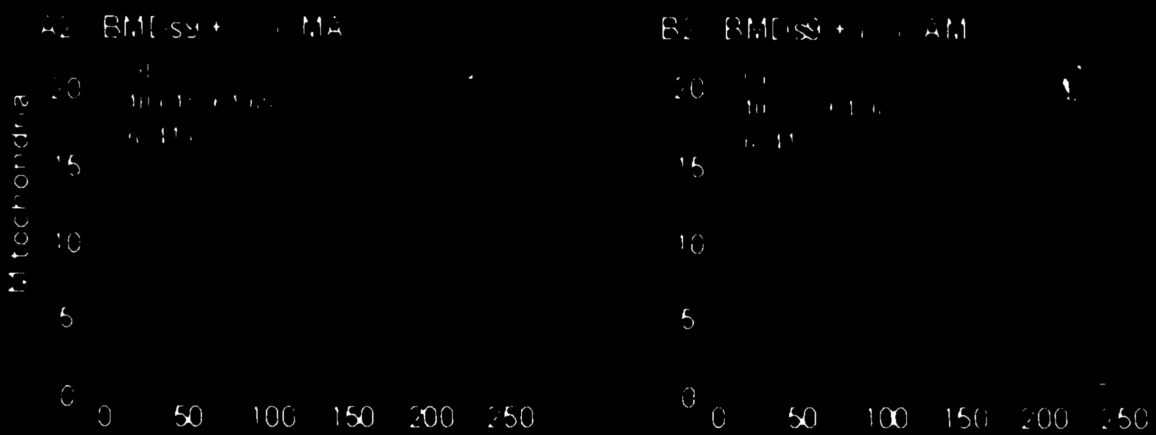


Figure 16

Table 8. Statistical testing of data for the mitochondrial membrane potential ($\Delta\Psi_M$) of oligodendroglial cells

Mann - Whitney U Test				T - test for independent samples			
Treatment	AST	PROL	OL	Treatment	AST	PROL	OL
BM / IM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$> 1.00 \times 10^{-15}$	BM / IM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$> 1.00 \times 10^{-15}$
BM-D9 / BM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	BM-D9 / BM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$
BM-D9 /IM	4.4×10^{-5}	2.6×10^{-7}	8.8×10^{-5}	BM-D9 /IM	4.9×10^{-5}	2.0×10^{-7}	3.6×10^{-14}
BM-DS9 /BM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	BM-DS9 /BM	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$	$>1.00 \times 10^{-15}$
BM-DS9 /IM	$> 1.00 \times 10^{-15}$	1.1×10^{-3}	8.8×10^{-14}	BM-DS9 /IM	$>1.0 \times 10^{-15}$	1.8×10^{-2}	6.9×10^{-1}
BM-D9 + Proad. / BM	—	—	4.23×10^{-13}	BM-D9 + Proad. / BM	—	—	1.2×10^{-10}
BM-Ds9 + Proad. / BM	—	—	$>1.00 \times 10^{-15}$	BM-Ds9 + Proad. / BM	—	—	$>1.00 \times 10^{-15}$
BM-D9 + Meta. / BM	—	—	2.9×10^{-1}	BM-D9 + Meta. / BM	—	—	7.7×10^{-1}
BM-Ds9 + Meta. / BM	—	—	$>1.00 \times 10^{-15}$	BM-Ds9 + Meta. / BM	—	—	$>1.00 \times 10^{-15}$
BM-D9 + P. But /BM	—	—	4.8×10^{-14}	BM-D9 + P. But /BM	—	—	9.5×10^{-1}
BM-Ds9 + P. But /BM	—	—	$>1.00 \times 10^{-15}$	BM-Ds9 + P. But /BM	—	—	$>1.00 \times 10^{-15}$
BM-D9 + MA. / BM	—	—	3.6×10^{-4}	BM-D9 + MA / BM	—	—	3.7×10^{-5}
BM-Ds9 + MA. / BM	—	—	6.4×10^{-1}	BM-Ds9 + MA / BM	—	—	1.0×10^{-1}
BM-D9 + AM / BM	—	—	2.0×10^{-1}	BM-D9 + AM / BM	—	—	2.0×10^{-1}
BM-Ds9 + AM / BM	—	—	1.3×10^{-1}	BM-Ds9 + AM / BM	—	—	4.7×10^{-1}

4.0 Discussion

4.1 An *In Vitro* Model System

This thesis describes for the first time the establishment of primary cultures which consists of over 60% differentiated oligodendroglial cells. The results of the present study are the first to report that differentiated cells of the oligodendroglial lineage like the PROLs and the OLs die by apoptosis upon withdrawal of insulin, IGF-I and serum.

There are certain advantages to the use of glial primary cultures. First, the purity of the cell populations derived from the primary mixed glial cultures is considerably higher when compared to the cell populations obtained from explants or reaggregate cultures. Second, mixed glial primary cultures offer the advantage of the presence of a stratified layer of O-2A progenitor cells over an astrocytic cell layer therefore purified populations of oligodendrocytes or astrocytes can be obtained from the same culture using a replating procedure.

O-2A progenitors can be isolated from the underlying astrocytic layer using mechanical shake-off method, by immunoisolation on solid substrates, or by fluorescence activated cell sorting. The isolated progenitors can be then developmentally synchronized to obtain cells at specific phenotypic stages. The use of primary cultures allows for the control of the microenvironment in which the primary cultured cells grow. Defined culture conditions facilitate the developmental synchronization of the O-2A progenitor differentiation. In addition, a controlled culture environment facilitates the identification and testing of the effects of trophic factors or other agents on oligodendroglial differentiation or survival.

An important finding of the studies utilizing *in vitro* models is that oligodendroglial cells in culture follow a schedule that approximates the timing of differentiation of the progenitor cells *in vivo* (Gard and Pfeiffer, 1989). Oligodendroglial cells are formed at birth and differentiate postnatally as opposed to neurons, which become established during embryogenesis. It is interesting to note that oligodendrocytes develop later than neuronal cells after the completion of axonal outgrowth. This late development has been postulated to occur because oligodendrocytes exert a strong inhibitory effect on axonal growth (Caroni and Schwab, 1988). Several proteins synthesized by oligodendrocytes including NI-35/250, bNI-220, myelin-associated glycoprotein, tenascin-R, and NG-2 have been described to inhibit neurite outgrowth (Spillmann *et al*., 1998).

The cerebral O-2A progenitor cells were first identified in a rat optic nerve model (Raff *et al*., 1983). The current study has utilized O-2A progenitor cells isolated from the cerebral cortices of the rat in its *in vitro* model system. Several other studies have used the rat cerebral cortex as a source for isolating O-2A progenitor cells instead of the optic nerve (Behar *et al*., 1988; Espinosa de los Monteros *et al*., 1988; Espinosa de los Monteros *et al*., 1986; McMorris *et al*., 1986). The use of the cerebral cortex offers the advantage of obtaining a higher yield of O-2A progenitor cells in comparison to the optic nerve.

4.2 Immunocytochemical and Morphological Cell Identification

The identification of oligodendroglial cell types on the sole basis of immunocytochemical data is not always practical by itself. This can be illustrated by using the example of the cell stage specific expression of a surface antigen, galactocerebroside (GC). Many earlier studies incorrectly interpreted the expression of GC to be linked to terminal differentiation and maturation of oligodendroglial cells. As the O-2A progenitor cells differentiate into the partially differentiated PROLs, the cells lose their reactivity to A2B5 and begin to express the surface antigen GC and an intracellular antigen, CNPase-I. Therefore, the expression of GC coincides with the appearance of PROLs rather than the OLs. The PROLs mature to form the fully differentiated OLs. Previous studies have shown that cells at this final stage of differentiation express MBP and PLP. Additionally, the differentiated cells also continue to express the PROL markers, GC and CNPase-I. Since there is a considerable overlap in the expression of antigens in the partially differentiated and fully differentiated oligodendroglial cells, it is necessary to combine the immunocytochemical observations with morphological features of these cells to correctly identify of the cell types found in the oligodendroglial lineage.

In the present study, positive identification of the individual cell types in the cultures was achieved using a combination of approaches including phase contrast microscopy, immunocytochemistry and methylene blue morphological staining. The PROLs were found to express GC and CNPase-I and had three to four primary processes with few secondary processes. The fully differentiated OLs however were seen to possess six to eight primary processes and numerous web-like secondary and tertiary processes

and also were immunopositive for antibodies that recognized MBP and PLP. The identification of MBP and PLP immunopositive cells as fully differentiated cells of the oligodendroglial lineage gains support from several studies which have observed that the MBP positive cells are incapable of any further cell division (Wood and Bunge, 1986). Apart from MBP and PLP, additional OL specific proteins have been characterized whose functions have not been completely elucidated. Included among the OL specific proteins are myelin-associated glycoprotein (Trapp, 1990), myelin/oligodendrocyte glycoprotein (Scolding et al., 1992) and myelin/oligodendrocyte specific protein (Dyer and Matthieu, 1994).

The correlation of the presence of immunocytochemical marker proteins to specific morphological changes in the cells, particularly changes in process morphology, allowed for the counting of changes in the numbers of specific cell types at a number of time points after insulin, IGF-I and serum withdrawal using a simple methylene blue staining process. Because of cell to cell variability in immunoreaction product, dependence on immunocytochemistry alone would have made the procedure considerably less reliable.

4.3 Insulin, IGF-I, Serum Withdrawal and Oligodendroglial Apoptosis

The *in vitro* model system of the present study demonstrates that insulin and IGF-I have the capacity to decrease apoptosis in O-2A progenitors, PROLs and OLs. Studies with oligodendrocytes in the rat optic nerve have revealed that oligodendroglial cells are overproduced during development (Barres *et al.*, 1992). Over half of the newly formed oligodendrocyte progenitor cells die due to lack of available trophic signals. Based on

their findings, Barres and co-workers hypothesized that survival / trophic factors ultimately decide the final numbers of myelin forming oligodendrocytes in any specific region of the CNS. Insulin and IGF-I, not only affect oligodendroglial differentiation and maturation but also contribute to the regulation of oligodendroglial cell survival (McMorris *et al.*, 1993).

It was previously shown that IGF-I and insulin decrease apoptosis in O-2A progenitors which was thought to be the point of regulation of OL numbers (Barres *et al.*, 1992). Data from the present study are the first to show that insulin and IGF-I can also decrease apoptosis of PROLs and OLs. These findings are consistent with previous studies showing that insulin can increase the numbers of oligodendrocytes grown in serum free conditions (Eccleston and Silberberg, 1984; Saneto and de Vellis, 1985). Other studies have shown that IGF-I produced by developing oligodendrocytes or astrocytes, acts additively with trophic factors like PDGF and NT- 3 to promote the proliferation and survival of oligodendrocyte precursor cells (Barres *et al.*, 1993). The present study suggests that similar actions may extend to fully differentiated OLs themselves and those actions may depend on an anti-apoptotic capacity of IGF-I and insulin on the OLs. In the model established in the present study, insulin, IGF-I and serum withdrawal is also accompanied by removal of 1% serum from the culture media. The data obtained on the anti-apoptotic effects of insulin and IGF-I on OLs in the present study could be strengthened by the addition of a 1% serum alone treatment condition. It is possible that the presence of 1% serum in the culture media could effect an increase in cell survival or conversely reduce apoptotic death but not to a similar extent brought about by the presence of insulin and IGF-I.

Very few studies have focused on glial cell apoptosis, more particularly on PROL and OL apoptosis. Previous studies have utilised cultures consisting of undifferentiated O-2A progenitor cells in order to study glial cell apoptosis. The first report showing a possible relationship between trophic factor withdrawal and an O-2A lineage cell apoptosis came from the studies of Barres *et al.* (1992). Their study demonstrated that newly formed oligodendroglial precursors die with morphological features suggestive of apoptosis when deprived of trophic support. Another study demonstrated that oligodendroglial progenitor cells isolated from the rat forebrain showed apoptotic cell death features upon removal of bFGF (Yasuda *et al.*, 1995). The presence of a combination of trophic factors may be essential to prevent oligodendroglial cell apoptosis *in vivo*.

The insulin, IGF-I and serum withdrawn oligodendroglial cells showed evidence for apoptotic nuclear degradation within 6 hours after insulin, IGF-I and serum withdrawal. Peak apoptotic death was observed at 18 hours after withdrawal of insulin, IGF-I and serum. The time course of apoptosis observed in the present study is similar to that of IGF-I deprived rat optic nerve O-2A progenitor cells which die within 18 hours (Barres *et al.*, 1992). Based upon those findings, it seems likely that after withdrawal of insulin and IGF-I, both undifferentiated O-2A progenitor cells and differentiated PROLs and OLs undergo apoptosis with a similar time course of death. However, the present studies suggested that IGF-I, insulin and serum withdrawal induced apoptotic death seems to be higher in the more differentiated cells of the oligodendroglial lineage.

The time course of apoptotic cell death can vary depending on the cell type and the insult that induces apoptosis. Apoptosis in neuronally differentiated PC12 cells has

been seen to begin between 6 and 12 hours after serum and NGF withdrawal (Wadia *et al.*, 1998). The apoptotic death of insulin and IGF-I deprived oligodendroglial cells appears to occur over a more protracted time course that of the serum and NGF deprived PC12 cells. The time course of apoptosis seems to depend on the intensity of the insult that induces the apoptosis (Hartley *et al.*, 1994), which may account for the longer time course of the oligodendroglial apoptosis induced by insulin, IGF-I and serum withdrawal.

IGF-I and insulin influence oligodendroglial survival by binding to their receptors IGFR and IR. Ligand binding activates the tyrosine kinase domain of the receptors resulting in receptor autophosphorylation and receptor activation. The activated receptors phosphorylate several substrate proteins like insulin receptor substrate (IRS)-1, and IRS-2 (Mendez *et al.*, 1996) on tyrosine residues that bind to Src homology-2 (SH2) proteins including Grb-2, crk, fyn, nck and PI-3 kinase (Yenush and White, 1997). Activated SH2 proteins like Grb-2 and PI-3 kinase are critical to further intracellular signaling and prevention of apoptotic death (D'Mello *et al.*, 1997). Grb-2 activates the $p21^{ras}$/ERK pathway by binding to the guanine nucleotide exchange factor SOS and facilitates the exchange of GDP for GTP. Activation of $p21^{ras}$ leads to the phosphorylation and activation of Raf-1. Phosphorylated Raf-1 activates downstream MAP kinases, which in turn activate extracellular signal-regulated kinases (ERKs). The ERKs translocate into the nucleus and regulate the synthesis of proteins that are critical to cell survival like BCL-2 and BCL-x_L. Feldman's group has suggested that activation of the IGFR maintains normal BCL-2 levels in human neuroblastoma cells while apoptotic death stimuli induced a four fold decrease in BCL-2 levels (Singleton et al., 1996). It is likely that on withdrawal of insulin and IGF-I, IR and IGFR mediated activation of the IRS

proteins would not occur. Absence of activated IRS proteins would result in inactivation of the MAP kinase pathway or the PI-3 kinase pathway that control the synthesis of key cell survival / anti-apoptotic proteins.

Oligodendroglial survival can also be mediated by activation of receptors other than IR and IGFR. Recently, a study demonstrated that activation of trkA NGF receptor could counter the apoptosis initiating effects of p75 nerve growth factor receptor (p75NGFR) and effect increases in oligodendroglial survival (Yoon *et al.*, 1998). Therefore the study of Yoon and co-workers (1998) suggests that signaling by ligand-activated trk receptors leading to cell survival plays a crucial role in maintaining the balance between cell viability and apoptosis in oligodendroglial cells.

4.4 IGF-I, Insulin and Regulation of Oligodendroglial Proteins

Insulin and IGF-I are considered to be involved in the regulation of myelin gene expression and myelin synthesis. In the present study, data obtained suggest that insulin and IGF-I regulate OL specific marker proteins like MBP and PLP. The western blot data presented in the present study showed a decrease in MBP in the oligodendroglial cells after insulin, IGF-I and serum withdrawal. The MBP result obtained is consistent with the nuclear and intact cell counts obtained in this study which show that withdrawal of insulin, IGF-I and serum decreased OL survival. Although part of the decrease in MBP can likely be accounted for by the greater proportion of death of OLs than other cell types in the cultures, it is also possible that decreased synthesis of the three proteins occurred on withdrawal of insulin, IGF-I and serum. Furthermore, the data also shows that agents like (-)-deprenyl and (-)-desmethyldeprenyl increase the levels of marker proteins for

OLs like MBP and PLP. (-)-deprenyl was also observed to induce a slight increase in the levels of the 26 kD splice variant of PLP, DM-20. This data seems to be consistent with the intact cell count data of this study which revealed increased survival of differentiated OLs in the presence of (-)-deprenyl.

Little is known about the functional roles of the four PROL and OL proteins. MBP and PLP together form 80% of the total myelin in the CNS. Decreased MBP content has been correlated to reduced survival of OLs. A recent study showed that partial transection of the spinal cord induced demyelination and OL cell death in the dorsal and ventral funiculi that was accompanied by downregulation of MBP mRNA (Bartholdi and Schwab, 1998). There have been recent reports on the involvement of PLP in mediating the survival of oligodendrocytes. In mice carrying mutations of PLP, there is a dramatic decrease in the numbers of oligodendrocytes as well as aberrant myelin formation (Jackson and Duncan, 1988). A recent study reported that in rats carrying a point mutation in exon III of the PLP gene, severe demyelination and OL cell death was observed (Lipsitz et al., 1998). Furthermore, the OLs in the rats with PLP mutations were found to be dying by apoptosis. This suggests that PLP is crucial for the survival and myelinating capabilities of oligodendroglial cells.

Paradoxically, other studies have offered evidence that overexpression of the PLP gene leads to OL cell death (Kagawa *et al.*, 1994). A very recent study has demonstrated that the perinuclear accumulation of PLP leads to the apoptotic death of OLs in a mice model of Pelizaeus-Merzbacher disease, a demyelinating disease occurring due to mutations, deletions or duplications of the PLP gene (Gow *et al.*, 1998). The findings of all these studies suggest that PLP may play a role in regulating OL survival.

4.5 Cell Density and Oligodendroglial Apoptosis

Although it is known that the absence of trophic factors, insulin and IGF-I decreases the survival of oligodendroglial cells, it is not known whether cell extrinsic factors like cell plating density regulates oligodendroglial apoptosis. The present study demonstrates that oligodendroglial cells underwent apoptosis, which was at least in part, mediated by cell plating density. At lower plating density, the number of apoptotic nuclei was approximately 15 fold higher in BM as opposed to IM. Higher plating density however increased the number of apoptotic nuclei in BM approximately 8.5 fold higher than in IM. This data suggests that oligodendroglial cells at lower plating density are more vulnerable to apoptosis induced by withdrawal of trophic support.

Lower plating densities have been shown to cause apoptotic cell death in several cell types. Lens epithelial cells, rat and chick chondrocytes, HL-60 cells, sciatic nerve fibroblasts and neutrophils die by apoptosis when cultured in low plating densities in the absence of serum or growth factors (Ishizaki *et al.*, 1994; Ishizaki *et al.*, 1995). A recent study reported that mature motoneurons died by apoptosis within 24 hours when cultured at low densities and in the absence of other cell types (Oorschot and McLennan, 1998). The findings of the motoneuron study of Oorschot and McLennan seem to be consistent with the findings of the present study that mature oligodendroglial cells die by apoptosis when cultured in low densities and therefore it can be suggested that mature neuronal and glial cells as opposed to undifferentiated cells of the nervous system show a propensity to undergo apoptotic death when cultured in low densities in the absence of serum or growth factors.

A number of possibilities emerge that could account for the decreased survival and increased apoptosis when cells are cultured at lower plating densities. First, it seems likely that an absence of extracellular survival promoting signals can decrease cell survival. Trophic factors such as PDGF, IGF-I, CNTF synthesized and released by astrocytes constitute some of the extracellular survival promoting molecules. It seems likely that most cells in the CNS require continuos signaling and the release of survival promoting molecules from other cells in order to prevent apoptosis. Secondly, at lower plating densities there might be a decrease in the levels of antioxidant molecules like glutathione and SOD-2, which would thereby facilitate the intracellular build up of oxidative radicals that can cause cell death by apoptosis. Lastly, lower plating densities might induce the release of apoptosis-initiating factor by a mechanism that would involve the activation of ICE like proteases, as described earlier. The released apoptosis initiating factors can induce apoptotic DNA fragmentation.

4.6 New Protein Synthesis and OL and PROL Apoptosis

As shown for the first time by the above studies using actinomycin D and cycloheximide, in a similar manner to the PC12 cell apoptosis caused by serum (Rukenstein *et al.*, 1991) or serum and NGF withdrawal (Tatton *et al.*, 1994) , oligodendroglial apoptosis induced by insulin, IGF-I and serum withdrawal is new protein synthesis independent. Those findings contrast with an earlier report that cycloheximide inhibited the cell death of O-2A progenitor cells hence O-2A cell death is new protein synthesis dependent (Barres *et al.*, 1992). Barres *et al.* (1992) hypothesized that oligodendroglial cells possess a suicide program that is activated when cells are deprived of essential trophic support. It is possible that newly formed oligodendroglial precursor cells, which were examined in that study, require the synthesis of new "death" proteins to complete the apoptotic process. The interpretation of the studies of Barres *et al.* (1992) has to be treated with caution since more recent studies have shown that cycloheximide protection of cells against a particular insult does not necessarily justify the conclusion that death genes or proteins are involved in the cell death process (Furukawa *et al.*, 1997). The study of Furukawa and coworkers has suggested increased survival in the presence of cycloheximide to result from an increase in the levels of key cell survival proteins like BCL-2. However, further differentiation into PROLs and OLs, as observed in the present study, may result in a modification of the apoptotic process so that newly synthesized death proteins are no longer required and the process depends on the relative levels of the constitutive proteins which mediate cell survival and death. The result obtained in the present study that OL apoptosis is independent of new protein synthesis is reliably

justified since the results were observed in the presence of a transcriptional blocker, actinomycin and a translational blocker, cycloheximide unlike the study of Barres and coworkers who have used only cycloheximide to block new protein synthesis.

4.7 (-) -Deprenyl and Increased Oligodendroglial Survival

The present study showed that (-) -deprenyl increased the survival of oligodendroglial cells which were deprived of insulin and IGF-I. The anti-apoptotic action of deprenyl seems to be limited to its (-) enantiomer (Ansari *et al.*, 1993) as has been shown for oligodendroglial cells in the present studies. (-) -Deprenyl inhibits MAO-B by forming a complex leading to the oxidation of (-) -deprenyl and reduction of the flavine adenine dinucleotide (FAD) moiety. (-) -Deprenyl and the reduced FAD moiety bind covalently (Ansari *et al.*, 1993). Since MAO-B possesses FAD binding domains, (-) -deprenyl in combination with reduced FAD binds to the enzyme non-covalently and inactivates it.

However, as discussed above, (-) -deprenyl mediates its survival enhancing action in an MAO-B independent manner (Tatton and Chalmers-Redman, 1996). Recent work has suggested that (-)-deprenyl-like compounds may reduce apoptosis by binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kragten *et al.*, 1998). Although GAPDH facilitates apoptosis in a variety of types of neurons (Ishitani and Chuang, 1996; Ishitani *et al.*, 1996; Ishitani *et al.*, 1996; Ishitani *et al.*, 1997; Sunaga *et al.*, 1995), it is not known whether it plays a role in oligodendroglial apoptosis.

The finding that (-)-deprenyl reduces oligodendroglial apoptosis suggest a possible role for GAPDH, if in fact, binding to GAPDH mediates (-)-deprenyl anti-

apoptosis. Recently, Kragten and coworkers have shown in rotenone exposed human neuroblastoma PAJU cell line that (-)-deprenyl and related compounds mediate, at least part of, their anti-apoptotic effects by binding to GAPDH (Kragten *et al.*, 1998). The P450 cytochrome blockade experiments in the present work are the first to establish that (-)-desmethyldeprenyl rather than (-)-deprenyl itself mediates the anti-apoptosis in the oligodendroglial cells. Experiments with three different general P450 cytochrome inhibitors, proadifen (SKF 525A), piperonyl butoxide and metapyrone, all led to the same findings: 1) that prevention of (-)-deprenyl metabolism by P450 cytochromes blocks the capacity of the compound to reduce oligodendroglial apoptosis and 2) prevention of the metabolism of (-)-desmethyldeprenyl by P450 cytochromes does impair the capacity of (-)-desmethyldeprenyl to reduce oligodendroglial apoptosis. Three P450 cytochrome inhibitors were required since most of the inhibitors have several sites of action. For example, proadifen and metapyrone inhibit P450 cytochromes and both also modulate bradykinin-releasable Ca^{2+}-pumping pools in cells (Graber *et al.*, 1997; Suarez-Kurtz and Bianchi, 1970). Modulation of the Ca^{2+} pools might be argued to be responsible for the blockade of anti-apoptosis by (-)-deprenyl rather than P450 cytochrome inhibition. Piperonyl butoxide also inhibits P450 cytochromes but does not share the Ca^{2+} action of the other two inhibitors (Benchaoui and McKellar, 1996). Accordingly, the use of three different inhibitors greatly strengthens the interpretation that cytochrome P450 mediated metabolism of (-)-deprenyl is necessary for its anti-apoptotic action. Since (-)-desmethyldeprenyl appears to be the active compound, it might bind to GAPDH, rather than (-)-deprenyl, and the binding may compromise the pro-apoptotic capacity of

GAPDH, thereby reducing apoptosis in the oligodendroglial cells. It is possible that (+)-desmethyldeprenyl may not bind to the protein, which would account for the stereospecificity of the anti-apoptotic action on the oligodendroglial cells.

The anti-apoptotic effects of (-) -deprenyl on neuronally differentiated PC12 cells has been attributed to a capacity to alter gene transcription and induce selective changes in new protein synthesis (Tatton and Chalmers Redman, 1996). It is likely that similar changes in new protein synthesis are induced by (-)-deprenyl or (-)-desmethyldeprenyl in the oligodendroglial cells, given the above findings which show that transcriptional or translational inhibition prevents either (-)-deprenyl or (-)-desmethyldeprenyl from reducing oligodendroglial apoptosis.

As detailed above, (-) -deprenyl treatment induces changes in the levels of a large number of proteins in different cell types, including increases in BCL-2 (Tatton *et al.*, 1994) . BCL-2 has been shown to be located, in part, in the outer membranes of mitochondria (Lithgow *et al.*, 1994; Riparbelli *et al.*, 1995), near to or at the peripheral benzodiazepine binding protein of the PTP (Carayon *et al.*, 1996). Kroemer and colleagues have shown that the presence of BCL-2 near the PTP serves to maintain PTP closure and maintain $\Delta\Psi_M$ (Marchetti *et al.*, 1996; Zamzami *et al.*, 1996). In early apoptosis, BCL-2 decreases in the mitochondrial membranes (Gillardon *et al.*, 1995), which results in PTP opening and likely contributes to the early apoptotic decrease in $\Delta\Psi_M$. Opening of the PTP, associated with the decrease in $\Delta\Psi_M$, results in the free exchange of solutes between the mitochondrial matrix and the cytosol, mitochondrial swelling and the escape of mitochondrial factors which induce the degradative phase of apoptosis (see Kroemer *et al.*, 1997). Mitochondrial BCL-2 has been shown to prevent

the release of pro-apoptotic factors from mitochondria (Susin *et al.*, 1996a; Yang *et al.*, 1997). Agents which maintain or increase cellular levels of BCL-2, like (-)-deprenyl, would therefore be expected to maintain $\Delta\Psi_M$ and prevent opening of the PTP leading to apoptotic degradation (see Wadia *et al.*, 1998).

GAPDH appears to promote apoptosis by down regulation of the increase in BCL-2 synthesis that accompanies cellular damage (Carlile, Chalmers-Redman, Borden and Tatton, personal communication). The above finding that (-)-deprenyl and (-)-desmethyldeprenyl maintain $\Delta\Psi_M$ in the oligodendroglial cells could be explained by the binding of (-) -desmethyldeprenyl to GAPDH leading to a maintenance of $\Delta\Psi_M$ with maintained closure of the PTP. Prevention of PTP opening would then result in a decrease in apoptosis in the cells. The validity of this model for the action of (-)-desmethyldeprenyl will have to be evaluated in a step by step manner in the oligodendroglial cells.

Rather than acting directly on the oligodendroglial cells to increase BCL-2 levels, (-)-desmethyldeprenyl may act through astroglial intermediaries. (-)-Deprenyl has been shown to increase the synthesis of CNTF, NGF and bFGF in astrocytes (Biagini *et al.*, 1994; Semkova *et al.*, 1996; Seniuk *et al.*, 1994). It has been suggested that increased availability of trophic support may account for the anti-apoptotic capacity of (-)-deprenyl (Koutsilieri *et al.*, 1996). A trophic basis for the decreased apoptosis could also explain the maintenance of $\Delta\Psi_M$ in the oligodendroglial cells since some trophic factors have been shown to increase $\Delta\Psi_M$ (Mattson *et al.*, 1993).

The present understanding of apoptosis is thought to be associated with a sequence of mitochondrial events involving opening of the PTP and a decrease in $\Delta\Psi_M$.

Although the studies of Wadia *et al*. (1998) as well as Paterson *et al*. (1998) make direct measurements of mitochondria to demonstrate a fall in the $\Delta\Psi_M$ in early apoptosis, it might be argued, that the decrease in $\Delta\Psi_M$ is simply a correlate of apoptotic death, but is not one of the steps leading to the death. As shown in my studies, the level of cell survival and $\Delta\Psi_M$ covary across a number of different treatments. That is, as shown in table 9, factors which increase or decrease survival have the same effect on $\Delta\Psi_M$. These findings strengthen the argument that decreases in $\Delta\Psi_M$ play a role in the genesis of oligodendroglial apoptosis.

Table 9. Relationship between $\Delta\Psi_M$ and OL cell survival.

	Survival (24 h)	$\Delta\Psi_M$ (18 h)
IM	++++	++++
BM	++	++
BM-D9	++++	+++
BM-Ds9	++++	++++
BM-D9 + Proad.	++	++
BM-D9 + Meta.	++	++
BM-D9 + P.But.	++	++
BM-Ds9 + Proad.	++++	++++
BM-Ds9 + Meta.	++++	++++
BM-Ds9 + P.But.	++++	++++
BM-D9 + MA	++	+
BM-D9 + AM	++	+
BM-Ds9 + MA	++	+
BM-Ds9 + AM	+	+

++++ = Control Levels

+++ = 60-90% of Control

++ = 30-60% of Control

+ = 0-30% of Control

4.8 Possible Therapeutic Benefits

This is the first study to show that (-)-desmethyldeprenyl can reduce PROL and OL apoptosis following withdrawal of insulin, IGF-I and serum. A number of studies have investigated into a variety of neurotrophic molecules that have the capacity to interrupt apoptotic cell death as possible agents for therapeutic benefits. As systemic therapeutic agents, the therapeutic approach involving neurotrophic molecules has a number of drawbacks, most prominently, their inability to cross the blood-brain barrier as well as initiate side effects by interacting with cells that are otherwise healthy and undamaged in the nervous system. However, agents like (-)-desmethyldeprenyl can not only diffuse easily into the nervous system but can also be highly specific in its interaction only with degenerating or damaged cells by its capacity to selectively alter gene expression and protein synthesis in those cells.

It is now known that OLs die via apoptosis in response to several death inducing stimuli including insufficiency of trophic support (Barres *et al*., 1992), exposure to various cytokines (Louis *et al*., 1993; Larocca *et al*., 1997). OL apoptosis has also been observed to occur in different pathological conditions like Alzheimer's diesease (Lassmann *et al*., 1995), in experimental allergic encephalomyelitis (Pender et al., 1991) and in the spinal cord white matter following spinal cord injury (Shuman *et al*., 1997). Since there is now evidence to show that OL apoptosis plays a role in the pathogenesis of MS (Dowling et al., 1997), agents like (-)-desmethyldeprenyl that reduce oligodendroglial apoptosis may be of therapeutic value in reducing the death of apoptotic OL cells. The reduction in OL apoptotic death by (-)-desmethyldeprenyl would occur by

diffusion of (-)-desmethyldeprenyl into the nervous system and its ability to increase the levels of key survival proteins like BCL-2 as well as binding to pro-apoptotic molecules like GAPDH thereby preventing GAPDH to effect apoptosis.

The findings of the present study on the maintenance of $\Delta\Psi_M$ by (-)-desmethyldeprenyl is of vital importance as such agents that maintain $\Delta\Psi_M$ and PTP closure may offer new and effective means of treating OL apoptosis. Lastly, (-)-desmethyldeprenyl would also prevent axonal loss in demyelinating conditions which would ensure the presence of sufficient amounts of trophic support for OL survival. These factors in combination would in turn slow the time course of the progression of MS as well as reducing the severity of the neurological deficits associated with MS and other pathological diseases.

5.0 Summary

The data presented in this thesis describes for the first time a primary culture model consisting of a high proportion of differentiated oligodendroglial cells. Using this model, the present study is the first to report that differentiated OLs die by apoptosis on withdrawal of insulin, IGF-I and serum. The study shows that (-)-deprenyl and its metabolite, (-)-desmethyldeprenyl can effectively reduce OL apoptotic death. It is known that the differentiated cells of the O-2A lineage, PROLs and OLs are targets of degeneration and death in demyelinating diseases of the CNS. This primary culture model allows for the study of the cell death process in differentiated PROLs and OLs as opposed to previous models, which have largely used O-2A progenitors. The study uses a number of measures to demonstrate the relationship between cellular loss and apoptotic nuclear degeneration of differentiated PROLs and OLs upon withdrawal of insulin and IGF-I. Specifically, apoptotic death was confirmed by using two independent *in situ* methods as well as DNA gel electrophoresis. Peak apoptotic cell death for OLs and PROLs was found to occur at 18h after withdrawal of IGF-I and insulin. Cell death also appeared to vary depending on the plating density of the cultures.

Apoptosis of the IGF-I and insulin withdrawn PROLs and OLs was significantly reduced by (-) -deprenyl. The (+) enantiomer of deprenyl did not reduce PROL and OL death showing that the actions of deprenyl is stereospecific. (-) -Deprenyl could reduce death of all the cell types of the O-2A lineage, but most particularly PROLs and OLs. This study is the first to report that a major metabolite of deprenyl, (-)-desmethyldeprenyl was also effective in reducing PROL and OL apoptosis. Blockade of

cytochrome P450 enzyme dependent metabolism of (-)-deprenyl but not of (-)-desmethyldeprenyl, established that (-)-desmethyldeprenyl was the active metabolite responsible for the anti-apoptotic effects of (-)-deprenyl. The other two major metabolites of (-)-deprenyl, (-) -methamphetamine and (-) -amphetamine did not improve cell survival after insulin, IGF-I and serum withdrawal but competitively inhibited the anti-apoptotic actions of (-)-deprenyl or (-)-desmethyldeprenyl. The anti-apoptotic effects of (-)-desmethyldeprenyl was found to be blocked by cycloheximide and actinomycin D showing that (-)-desmethyldeprenyl was dependent on the induction of new protein synthesis for its anti-apoptotic action. Furthermore, Western blot analysis of protein levels revealed that insulin, IGF-I and serum withdrawal decreased the levels of OL proteins like MBP but not of PLP.

The present study demonstrated that withdrawal of IGF-I and insulin shifted the mitochondrial CMTMR fluorescence intensity distributions to lower values, which represented decreases in $\Delta\Psi_M$ in the OLs, PROLs and ASTs. (-)-Deprenyl and its major metabolite (-) -desmethyldeprenyl prevented the shift of the distributions to lower values. Furthermore, experiments with cytochrome P450 inhibitors showed that the effects of (-)-deprenyl on $\Delta\Psi_M$ could be blocked but not that of (-)-desmethyldeprenyl. The other metabolites of (-)-deprenyl, (-) -methamphetamine and (-)-amphetamine, at high concentrations, shifted the mitochondrial fluorescence distributions to lower values showing a blockade of the effects of both (-)-deprenyl and (-)-desmethyldeprenyl on $\Delta\Psi_M$. These results demonstrate for the first time the mitochondrial participation in insulin, IGF-I and serum withdrawal induced apoptosis of

OLs and PROLs, and that maintenance of $\Delta\Psi_M$ is critical to the survival of the differentiated PROLs and OLs.

6.0 References

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